PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In application of

Ryuichi MORISHITA , et al.

Docket No: Q64360

Appln. No.: 09/856,374

Confirmation No.: 8301

Group Art Unit: 1632

Filed: May 21, 2001

Examiner: Qian Janice LI

For: GENE THERAPY FOR CEREBROVASCULAR DISORDERS

STATEMENT OF SUBSTANCE OF INTERVIEW

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Please review and enter the following remarks summarizing the interview conducted on June 7, 2004:

REMARKS

An Examiner's Interview Summary Record (PTO-413) was attached with the Office Action dated June 15, 2004.

During the interview, the following was discussed:

1. Brief description of exhibits or demonstration:

   None

2. Identification of claims discussed:

   Claims 13-17.

3. Identification of art discussed:

Isner et al., Morishita et al., and Ghodsi et al., all of record.

4. Identification of principal proposed amendments:

None.

5. Brief Identification of principal arguments:

The Examiner contended that although the amendment filed April 6, 2004 and the declaration filed May 11, 2004 did provide some valuable arguments and evidence regarding the patentability of the present invention, she maintains her position that the invention is obvious over the prior art. The Examiner reiterated her contention that because gene therapy using HGF and VEGF is known to have therapeutic effects in other organs, and gene therapy in general has been shown to be effective in treating brain disease, the invention is obvious.

6. Indication of other pertinent matters discussed:

The Examiner stated that the next Office Action would be non-final and provide more detailed reasoning in support of her position.

7. Results of Interview:

None.

It is believed that no petition or fee is required. However, if the USPTO deems otherwise, Applicant hereby petitions for any extension of time which may be required to

maintain the pendency of this case, and any required fee, except for the Issue Fee, for such extension is to be charged to Deposit Account No. 19-4880.

Respectfully submitted,

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WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: September 15, 2004

Gene Therapy for Human Malignant Brain Tumors

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PURPOSE

Brain tumors were the first human malignancy to be targeted by therapeutic transfer of nucleic acids into somatic cells, a process also known as gene therapy. Malignant brain tumor cells in the adult brain have some unique biologic features, such as high mitotic activity on an essentially postmitotic background and virtually no tumor spread outside of the central nervous system. Brain tumors seem therefore to offer major advantages in the design of tumor-selective gene therapy strategies, and the role of gene therapy in malignant glioma has been investigated since the late 1980s, initially in numerous laboratory studies and later on in clinical trials.

DESIGN

Retrovirus has been one of the earliest recombinant virus vectors used in brain tumors. Experiments in cell culture and in animal models have demonstrated the feasibility of retrovirus-mediated transduction and subsequent killing of glioma cells by toxic transgenes. Phase I and II clinical studies in patients with recurrent malignant glioma have shown a favorable safety profile and some efficacy of retrovirus-mediated gene therapy. However, the only prospective, randomized, phase III clinical study of retrovirus gene therapy in primary malignant glioma failed to demonstrate significant extension of progression-free or overall survival. Adenovirus- and herpes simplex virus type 1–based vectors have been actively investigated along with retrovirus, but their clinical use is still limited, mostly because of safety concerns. To increase efficacy, novel generations of therapeutic adenovirus and herpes simplex virus type 1 rely more on genetically engineered and tumor-selective lytic properties and less on the actual transfer of therapeutic genes.

CONCLUSIONS

The failure of most clinical gene therapy protocols to produce a significant and unequivocal benefit to brain tumor patients seems to be mainly due to the low tumor cell transduction rates observed in vivo, but it may also depend on the respective physical delivery strategy of the vector. Standard radiologic criteria for assessing the efficacy of clinical treatments may also not be fully applicable to the specific metabolic changes and blood-brain barrier permeability phenomena caused in brain tumors by virus-mediated gene therapy. Clinical trials in malignant glioma have nevertheless produced a substantial amount of data and have contributed to the continuous improvement of vector systems, delivery methods, and clinical protocols1. *(Cancer J 2003;9:180–188)*

KEY WORDS

Adenovirus, brain tumor, gene therapy, glioma, herpes simplex virus, liposomes, retrovirus

Malignant brain tumors were one of the earliest targets for human gene therapy, a strategy that uses the transfer of genetic material into somatic cells for therapeutic purposes.[1] Brain tumor cells represent islands of high mitotic activity on the background of a mostly postmitotic environment, such as the adult brain, and seem therefore to offer biologic targets, thereby allowing treatments with selective tumor toxicity to be designed.[2,3] From its beginnings, gene therapy in brain tumors was seen as an adjunct to proven cytoreductive and locally destructive methods, such as surgery and radiation therapy.[2,4]

The susceptibility of malignant brain tumors of glial origin (gliomas) to gene therapy has been investigated since the late 1980s. The initially favored and best-explored gene therapy approach included insertion of a genetic sequence into tumor cells, which renders these and their clonal progeny differentially sensitive to drug treatment.[5] The transgene/vector system that has been most widely used in the past decade was the herpes simplex virus thymidine kinase (HSV-*tk*) gene transferred by a replication-incompetent retrovirus (RV) vector, which is released in situ by fibroblast-derived RV vector-producing cells (RV-VPCs).[6–8] The early work on RV vectors instigated most of the current developments of novel generations of viruses for human gene therapy.

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Received on December 9, 2002; accepted for publication January 28, 2003.

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this article.

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Although at present, there is an impressive variety of different gene therapy strategies for the experimental treatment of brain tumors,[2,4,9] their therapeutic success is still very limited and is highly dependent on the qualities and limitations of the available vector system. Clinical trials to date have mainly focused on phase I studies for evaluation of toxicity and maximum tolerated dose.

Early clinical trials using RV indicated that the transfer of therapeutic genes to tumor cells within the brain may be met with multiple obstacles, and that RVs are essentially unable to achieve a useful tumor transduction rate. This made necessary the development of augmenting strategies (e.g., combining radiation therapy or chemotherapy with gene therapy) and immune response–activating approaches (e.g., using immunostimulatory cytokines).[10–13] Although, gene therapy for brain tumors is reasonably safe, concerns still exist regarding the delivery of unwanted vector-related genes and the potential pathological response of the brain.

VIRUSES USED AS VECTORS AND AS ONCOLYTIC AGENTS

Viruses are highly efficient vectors for transgene delivery to cells and may have therapeutic potential in their own right, apart from the transferred genetic sequences. Most of the virus vectors used for human gene therapy have been derived from wild-type viruses and have been extensively genetically engineered to create or enhance desirable features and remove undesirable ones, such as strong immunogenicity. There are two types of recombinant virus vectors: (1) those that are able to infect and enter tumor cells but are unable to replicate (replication deficient) and (2) those that are able to do so under certain circumstances, such as in mitotically active cells only (replication conditional).[1,2] Nonreplicating virus vectors are generally able to carry out efficient gene delivery and are biologically safe, but they usually do not produce toxicity to tumor cells on their own. Conditionally replicating virus vectors, such as herpes simplex virus type 1 (HSV) and adenovirus (AV), have been increasingly used in the treatment of brain tumors.[2,14,15] They are engineered to replicate in mitotic cells only (e.g., HSV) or in cells lacking functional tumor-suppressor proteins such as p53 or Rb (e.g., AV[14]) and are also known as oncolytic viruses. Treatment of tumors with oncolytic viruses may benefit from secondary production and release of virus from lysed tumor cells, which increases the initial titers and spreads the virus over a larger area.[15]

RETROVIRUS

RV-mediated gene transfer is particularly applicable to brain tumors because of the selectivity of nonreplicating recombinant RV for mitotic cells. In the brain, such mitotic cells are only the cells in a rapidly growing tumor, the proliferating activated endothelial cells in the neoplastic capillaries, and some reactive astrocytes.[3,16] When compared with other types of vectors, such as HSV and AV, RV vectors are more selective in terms of transgene delivery, and the transgene is inherited by all progeny cells of the infected cells.[17,18] Retrovirus vectors also show very low toxicity to normal brain tissue.[19–22] Despite these advantages, low titers (typically 1 x 10^5–10^7 infectious RV particles/mL) and instability of the virus particles, making the use of RV producer cell lines (packaging cells) necessary, have limited the clinical usefulness of RV vectors.[23] A major limitation for the transduction efficiency of first-generation recombinant RV vector was the inability to replicate, which on the other hand is a safety feature and was therefore deemed essential in human clinical trials.[21,22,24]

Clinical Studies with Retrovirus

The first clinical study with stereotactic intratumoral inoculation of RV-VPC in patients with recurrent malignant brain tumors achieved some promising results in terms of antitumor efficacy (Table 1). It was not, however, a controlled and randomized protocol.[25] In two subsequent studies in patients with recurrent GBM, HSV thymidine kinase (TK) and ganciclovir (GCV) gene therapy was performed by locally administered RV-VPC inoculated manually during open surgical resection of tumor.[22,26] In both studies, there were individual cases of extended recurrence-free periods, including one patient each with apparently arrested tumor progression for 2 years or longer. The authors of both reports independently concluded that the RV-VPC gene therapy strategy appeared to be adequately safe and effective to allow further clinical studies. The same conclusions were drawn by other investigators studying RV-VPC application in patients with recurrent glioblastoma multiforme (GBM).[27,28] A phase I study using intraoperative RV-VPC administration was carried out in pediatric recurrent malignant glioma.[29] Because no control group was included in the study, it was impossible to provide evidence for a significant gene therapy-mediated antitumor effect, although these authors[29] and later Kramm et al[30] described single cases of long-term survivors after gene therapy.

According to the evidence gathered in the aforementioned trials, a large prospective, randomized, and controlled phase III study seemed necessary for an ultimate confirmation of efficacy of this approach. To include as many patients as possible, the phase III protocol for RV-VPC–mediated gene therapy was used as an adjuvant to the standard therapy of maximum surgical resection and irradiation for newly diagnosed, previously un-

TABLE 1 Clinical Gene Therapy Trials in Human Glioma[a]

Author	Year	Tumor Type	Vector Type	Number of Patients	Study Phase	Significant Tumor Response
Izquierdo et al[28]	1996	Recurrent GBM	RV	5	I	N/A
Izquierdo et al[28]	1997	Recurrent GBM	RV	2	I	Yes
Colombo et al[37]	1997	Recurrent GBM	RV	4	I	N/A
Ram et al[25]	1997	Recurrent GBM	RV	15	I/II	Yes
Puumalainen et al[32]	1998	Recurrent GBM	RV	10	I	N/A
Klatzmann et al[26]	1998	Recurrent GBM	RV	12	I	Yes
Palu et al[10]	1999	Recurrent GBM	RV	4	I	N/A
Shand et al[22]	1999	Recurrent GBM	RV	48	II	Yes
Packer et al[29]	2000	Recurrent GBM	RV	12	I	N/A
Rainov et al[24]	2000	Primary GBM	RV	248	III	None
Harsh et al[33]	2000	Recurrent GBM	RV	5	I	N/A
Sandmair et al[27]	2000	Primary GBM	RV[b]	7	I	None
Rampling et al[40]	2000	Recurrent GBM	HSV	9	I	N/A
Markert et al[42]	2000	Recurrent GBM	HSV	21	I	Yes
Trask et al[46]	2000	Recurrent GBM	AV	13	I	Yes
Sandmair et al[27]	2000	Primary GBM	AV[b]	7	I	Yes
Jacobs et al[51]	2001	Recurrent GBM	Lipoplex	5	I	N/A
Kramm et al[30]	2002	Recurrent ependymoma	RV	1	Case study	N/A
Valery et al[67]	2002	Recurrent GBM	RV	1	Case study	Yes

Abbreviations: GBM, glioblastoma multiforme; N/A, data not available; lipoplex, cationic liposome/plasmid DNA complex; AV, adenovirus; HSV, herpes simplex virus; RV, retrovirus.

[a]Most of the studies were uncontrolled phase I or II protocols, and tumor response is therefore largely a subjective assessment not based on statistical comparison with a control group.

[b]Comparative study with both RV and AV vectors.

treated GBM.[24] Primary tumors were expected to respond better to gene therapy than most recurrent tumors investigated in previous studies. Radiation therapy and gene therapy (GCV.administration) overlapped partly to benefit from the alleged synergism of these modalities.[13,31] After 4 years of clinical and laboratory follow-up of the 248 patients with primary GBM, who were evenly divided in a gene therapy arm and a control arm, survival analysis was not able to demonstrate that the addition of gene therapy provided any advantage in terms of time to tumor progression or overall survival time.[24]

A common and severe drawback of all of these clinical protocols, which was recognized only retrospectively, was the fact that the overall transduction rate of brain tumor cells was very low. This was confirmed later in smaller and more specialized studies in human GBM employing marker genes, such as *lacZ*.[32]

To overcome the existing limitations and to achieve a significant effect against tumors in vivo, Palu et al[10] used RV vectors in a combined HSV-TK and cytokine (interleukin-2) gene therapy approach, speculating that interleukin-2 transgene expression even in a few tumor cells may elicit a systemic antitumor response and could result in major antitumor toxicity that is mediated by the host cellular immune response.[10] Their study was not controlled, and therefore, it was impossible to provide reliable data for the improved anti-tumor effect of this approach.

Even without any proven benefit to GBM patients, RV gene therapy itself, such as the grafting of RV-VPC in the walls of a tumor resection cavity in the brain, was not associated with major side effects or survival disadvantages. No increased incidence of postoperative neurologic complications was noted, and no acute inflammation or meningitis was observed in any of the gene therapy cases.[22,24–26] There were only two adverse events (seizures, hydrocephalus), possibly due to the gene therapy, in a series of 48 adults[22] and none in another series of 12 adults.[26] Harsh et al[33] administered RV-VPC by stereotactic injection in 5 patients with recurrent GBM, resecting the tumors 5 days later while reinjecting the tumor bed with RV-VPC. These authors described no gene therapy–related adverse events, although one patient experienced a lethal brain abscess after open surgery. The published results with murine RV-VPC seem to confirm the hypothesis that injection of xenogeneic cells in the tumor margin and the sur-

rounding mostly normal brain tissue does not cause significant clinical morbidity within the follow-up time frame (1–2 years). Compared with chemotherapy for malignant brain tumors, RV-mediated *HSV-tk*/GCV gene therapy appears to offer a better clinical safety profile with regard to the incidence of adverse events with chemotherapy.[34,35]

RV-mediated gene therapy was proved to be safe not only in adult patients but also in children, in whom the biologic background and the clinical presentation of malignant brain tumors are considerably different. Packer et al[29] treated 12 pediatric recurrent brain tumors and reported transient adverse events (seizures and signs of elevated intracerebral pressure) after RV-VPC injection in four patients.

Biosafety monitoring is an important part of every clinical study with virus vectors. So far, follow-up assays have been able to exclude any gain of replication competence through recombination of the vector with wild-type RV. The presence of transduced blood cells in the systemic blood circulation in a small number of patients presents a mostly theoretical risk and should therefore not be considered an unacceptable safety risk in the treatment of an otherwise rapidly fatal cancer.[21,22,36] Absence of a humoral response to the vector core protein in a phase III clinical study[24] suggests that the RV vector itself is not entering the systemic circulation, although antibodies to RV core protein have been shown previously in a small number of patients, particularly in those receiving multiple administrations of RV-VPC suspension.[21,25]

In Vivo Transduction Efficiency of Retrovirus

The principal requirement for the success of gene therapy with replication-incompetent virus vectors seems to be the achievement of adequate transduction levels of a functional transgene in target tumor cells. Because of the direct pharmacologic bystander effect and the indirect systemic anti-tumor immune response, transduction of 100% of the existing tumor cells is not a prerequisite for successful tumor ablation.[1,2,18]

Despite the considerable number of brain tumor patients receiving RV-VPC gene therapy to date, surprisingly little is known about the tumor transduction rate in vivo, except that it is undoubtedly very low. Harsh et al[33] investigated brain tumor tissue by quantitation of transgene (*neo*^{r}) levels in five patients, including the postmortem investigation of a patient 3 weeks after vector-producing cell injection. These authors reported consistent tumor transduction rates of < 0.002% in all cases. There was no evidence for the production of the transgene protein HSV-TK in resected tumor tissue, as detected by immunohistochemistry with an anti–HSV-TK antibody. A somewhat elevated HSV-TK enzyme activity was noted in RV-VPC–injected tumor samples from two patients, but this was measured by a highly sensitive radioactive kinase assay employing ^{3}H-labeled GCV. In any case, HSV-*tk* activity in human GBM samples was very low compared with transduction rates reported in successful control animal experiments.[33]

In a previous series that included 51 postgene therapy brain tumor biopsies or postmortem samples, 39 of which were proved positive for vector DNA by polymerase chain reaction, the highest transduction contained less than level (*neo*^{r} quantitation) found in two patients was 2.6%.[36] Most of the tumor specimens contained less than 0.03% transduced tumor cells. Other investigators have reported transgene expression for up to 7 days after intratumoral RV-VPC injection but were unable to differentially describe percentages of transduced tumor cells.[24,25,32]

In a gene marking study in human GBM patients, Puumalainen et al[32] injected RV or AV vector suspension in 10 patients with malignant glioma via an intratumoral catheter.[32] RV was repeatedly injected on 3 consecutive days into the tumor, followed by surgical resection a few days later. Gene transfer efficiency varied between < 0.01% and 4% with RV, and between <0.01% and 11% with AV. The transgene activity with both virus vectors was not evenly distributed throughout the tumor. Both glioma cells and endothelium in the neoplastic blood vessels were transduced with RV, but not with AV.[32] In an early study, Colombo et al[37] treated four recurrent GBM patients with stereotactic intratumoral injections of RV producer cells. The therapeutic transgenes used were *IL-2* and *HSV-tk*. The authors stated that transgene expression in tumor cells was confirmed by immunohistochemistry, although they did not quantify expression and related it to the amount of delivered RV producer cells.

HERPES SIMPLEX TYPE 1 VIRUS

Among other human viruses, HSV offers the opportunity to influence the replication of tumor cells directly within the central nervous system (CNS). The propensity of recombinant HSV vectors to replicate in tumor cells and the large transgene capacity of the virus provide an excellent experimental platform for the development of novel therapeutics.[1,2,17] In most preclinical and human studies, replication-conditional HSV vectors have been used which carry mutations in nonessential genes required for viral propagation in quiescent cells, but not in dividing cells, which can compensate missing virus functions through intrinsic cellular proteins that are active during cell proliferation.[15]

When used for treating brain tumors in animal models, replication-conditional HSVs propagate selectively in tumor cells, sparing surrounding neurons and nonre-

active glia from cytopathologic effects. Selective oncolytic viral replication in tumor cells in the brain provides some therapeutic advantages, such as increasing virus titers generated on site and lysis of permissive tumor cells without the use of an additional toxic transgene/prodrug system.[39] In addition, expression of HSV antigens elicited a systemic immune response with the potential for recognizing tumor antigens.[15]

Nearly all replication-conditional HSVs used for brain tumor gene therapy carry mutations in one or two of several different viral genes, such as TK, ribonucleotide reductase, uridine triphosphatase, or the neurovirulence factor γ34.5. Single gene deletions raise the risk of fatal encephalitis caused by recombinational reconstitution of latent wild-type HSV; double mutants may be safer, but they appear less toxic toward tumor cells.[38,39]

Rampling et al[40] carried out a clinical study to evaluate the biologic safety of the oncolytic HSV mutant 1716 in patients with relapsed malignant glioma. Direct intratumoral inoculation of doses up to 1×10^5 infectious particles (plaque-forming units [pfu]) was carried out, and no induction of encephalitis, no adverse clinical symptoms, and no reactivation of latent HSV occurred. A total of nine patients were treated, and apparently, tumor progression was controlled with some efficacy.[40]

Harland et al[41] further investigated the mutant HSV1716 virus in glioma patients. These authors demonstrated that HSV1716 is nontoxic when it is delivered to tumor or into brain adjacent to tumor, yet it replicates within tumor cells. They demonstrated that HSV1716 can persist in human glioma and has the potential to kill tumor cells over a prolonged period of time.[41]

Markert et al[42] used G207, a conditionally replicating oncolytic HSV derivative with deletions of both γ34.5 loci and an *lacZ* insertion disabling the *UL39* gene, for inoculation in human malignant glioma. The G207 mutant is hypersensitive to GCV and acyclovir, which represents an additional safety feature in cases of HSV encephalitis. Because G207 is replication deficient at temperatures greater than 39.5°C, the development of significant fevers, such as occurs in encephalitis, should be able to halt the spread of virus infection.

The G207 dose escalation study involved 21 patients and started with 1×10^6 pfu inoculated at a single enhancing tumor site. Three patients were enrolled at each dose level. The highest dose used in the escalation was 3×10^9 pfu at five tumor sites. No toxicity or serious adverse events could unequivocally be ascribed to G207. No patient experienced HSV encephalitis. Some radiographic and neuropathologic evidence was suggestive of anti-tumor activity, and long-term presence of virus DNA was proved in some cases.[42] No claims of efficacy are possible in such an early (phase I) trial, and planned future studies with these and other HSV mutants need to establish a true benefit to the patient.[39]

Important issues currently under consideration to improve the efficacy and safety of HSV therapy include evaluation of therapeutic transgenes, such as using toxicity-generating or -immunostimulating genes, improving vector delivery, and increasing the specificity of targeting virus to tumor cells. Preclinical studies evaluating the combination of HSV-mediated oncolytic therapy with gene therapy seem to have yielded promising results and may be the next step in the experimental virus treatment of malignant brain tumors.[1,15,39]

ADENOVIRUS

AV vectors are gaining importance in clinical trials for treatment of cancer. AV are nonenveloped, double-stranded, linear DNA viruses that may cause benign respiratory tract infections in humans. From the more than 49 different serotypes known to infect humans, serotypes 2 and 5 have been mainly developed as vectors because of their low pathogenicity and well-known structure.[4,14] Features contributing to the increasing therapeutic use of AV are high titers, structural stability, broad infectivity of dividing and nondividing cells, and high levels of transgene expression.[43] AVs are used at present mostly as robust gene carriers, but there are also naturally occurring or genetically engineered mutants known for their oncolytic activity, such as ONYX015 and others.[14] Early-generation AVs tend to generate a strong inflammatory response in vivo, which on the one hand may result in local side effects, and on the other hand may severely limit infection rates and virus titers with repeated application.[15]

Protocols for phase I trials in recurrent or progressive malignant glioma have been published that use recombinant AV–expressing human interferon-beta[44] or the *HSV-tk* gene.[45] These protocols are still open for enrollment, but no published information is available on the outcomes and side effects of the treatments.

Trask et al[46] treated 13 patients with recurrent malignant brain tumors with a single intratumoral injection of 1×10^8 to 1×10^{11} infectious particles of a replication-defective AV carrying the *HSV-tk* gene. Only patients who have received the highest dose, 1×10^{11} infectious particles, have shown central nervous system toxicity displayed as confusion, hyponatremia, and epileptic seizures. One patient survived 2.5 years after treatment and was in a stable condition. Two patients survived for 2 years each before lethal tumor progression. Ten patients died within 10 months of treatment, most of them from tumor progression. Neuropathologic examination of postmortem tissue demonstrated cavitation at the injection site, intratumoral foci of coagulative necrosis, and varying degree of infiltration of the residual tumor with macrophages and lymphocytes. Some evidence for ex-

tended control of tumor progression by AVs has been obtained in this noncontrolled study.[46]

A few other protocols are currently open in Europe and the United States and are actively enrolling patients for treatment with the oncolytic mutant ONYX015, a conditionally replicating E1B-deleted AV. It was initially believed that ONYX015 selectively lyses cells with dysfunctional or absent wild-type p53 protein.[47] However, later research showed that the virus is selective for neoplastic cells rather than for every type of p53-dysfunctional cell.[14,15]

Although AVs seem to be well adaptable to brain tumor therapy and have shown promising results in the few clinical studies, there is some reluctance to conduct advanced clinical studies that are designed to prove efficacy and biosafety, partly because late side effects due to chronic inflammation in the brain are suspected.[48]

NONVIRUS VECTORS

Nonvirus vectors have been shown to result in significant transduction rates of tumors in preclinical experimental studies, although they are generally less efficient than virus vectors.[49] The most frequently used nonvirus vectors are complexes consisting of cationic liposomes and plasmid DNA.[50] Mainly for reasons of efficacy of gene transfer and because of transgene delivery issues, liposomes have not been used in clinical trials in brain tumors, with the exception of one study. Jacobs et al[51] carried out a phase I clinical gene therapy trial for recurrent GBM in five patients. For transduction of the *HSV-tk* gene, these authors used a cationic liposome/plasmid complex. Positron emission tomography with ^{124}I-labeled synthetic GCV analogues was able to demonstrate *HSV-tk* gene expression in vivo and seemed to predict therapeutic response. No reliable evidence for the efficacy of the treatment was available in this early and mostly imaging-centered study. A recently published clinical protocol[52] seems to propose the continuation of these nonvirus vector studies in human tumors.

CURRENT CLINICAL PROTOCOLS FOR GENE THERAPY OF BRAIN TUMORS (NCI STUDY DATABASE)

Of the 114 open and active studies in adult brain tumor patients listed in the National Cancer Institute (NCI) database (www.nci.nih.gov), only two are bona fide gene therapy studies. Phase I/II study of intracerebral HSV (G207) in patients with recurrent malignant glioma (protocol NCI-V02-1695) is designed to determine the maximum tolerated dose of intracerebral G207. A secondary endpoint of the study is anti-tumor efficacy.

A phase I study of intratumoral AV encoding human interferon-beta in patients with recurrent or progressive glioblastoma (protocol NCI-V02-1696) also attempts to determine the maximum tolerated dose in these patients. A secondary outcome measure is represented by the 6-month tumor progression–free survival rate.

Delivery Modes

Efficient gene delivery is the first and most important requirement for successful treatment of brain tumors using the gene therapy paradigm. All previous clinical protocols called for direct manual or stereotactic injection of small volumes of vector suspension into the walls of a postsurgical tumor resection cavity or into an existing tumor mass.[22,26,28] Besides the technically virtually impossible task of standardizing distances between injection tracks and applied volumes, reflux of vector suspension from needle tracks is invariably encountered with the manual technique.[9] Stereotactic injections are less prone to error but are technically very demanding and are unlikely to establish themselves in routine use.

The route of virus vector administration has been demonstrated to affect both tumor transduction efficiency and spatial distribution, as well as the extent of transgene expression in invasive tumor cells and in the surrounding normal brain.[53,54] Three main modes of vector delivery to experimental brain tumors have been extensively studied in animal models and, in some cases, in clinical trials: stereotactic intratumoral inoculation of virus particle suspension or vector-producing cells; intrathecal and intraventricular injection of virus or vector-producing cells, and more recently, intravascular application of virus vectors.[9] The study of the modes of application and the factors that limit vector distribution and propagation in a brain tumor is of great importance to the improvement of present gene therapy strategies and to the development of more efficient approaches.[54]

LESSONS LEARNED FROM CLINICAL TRIALS ON GENE THERAPY

The greatest shortcoming of all clinical protocols for malignant brain tumors seems to be the low efficiency of tumor cell killing in situ, which in turn results from limited spatial distribution of vectors that prevent eradication of invading tumor cells at a distance from the main mass, as well as from less-than-lethal toxicity to tumor cells. In addition, the present inability to quantify levels or functional transgene activity in tumors prevents a meaningful correlation of transduction rates with vector delivery modes and with toxicity generation. To quantify vector-mediated tumor transduction, minimally invasive imaging must be included in future protocols, and some experimental studies already suggest that positron emission tomographic imaging and quantita-

tion of prodrug metabolism are feasible in vivo.[51,55,56] Quantitative or semiquantitative imaging of transgene expression in a tumor and in the surrounding brain tissue would ensure that the expected biologic activity is indeed available in vivo.

Further optimization of vectors, especially of targeted viruses, may also be essential for the improvement of clinical efficacy of gene therapy.[15] Recent developments, such as hybrid vectors, such as those based on HSV amplicons and retaining RV or AV features, may dramatically increase virus availability in a large human brain tumor and may allow for controlled replication of virus and therefore for a secondary spread of infection throughout the tumor.[57]

Combination of gene therapy with currently existing and established antineoplastic treatments, such as chemotherapy radiation therapy, may considerably increase the tumor selective toxicity without causing potentiation of side effects or unwanted toxicity to normal cells.[58] Furthermore, diffusion of prodrugs such as GCV into a brain tumor may vary according to the permeability of the tumor and the peritumoral tissue. Administration of GCV along a blood–tumor barrier modifier, such as the selective B2 bradykinin agonist labradimil (Cereport, Alkermes, Inc., Cambridge, MA) could result in a significantly higher intratumor and peritumor GCV concentration and subsequent elimination of persisting transduced cells.[59,60]

Many preclinical and a few clinical studies have shown that virus infection and destruction of brain tumor cells in vivo is accompanied by a systemic immune response against virus proteins and also against autologous tumor cells that involves T cells and natural killer cells.[61–65] This immunologic effect could be enhanced through the transfer of additional genes coding for immunostimulatory cytokines, such as interleukin-2[10] and interleukin-12.[66]

CONCLUSIONS

Gene therapy is a highly complex biologic paradigm for the treatment of brain tumors. Despite more than a decade of experience from numerous animal studies and human trials, it still seems to be at its very early stages of clinical development and implementation in therapeutic algo rhythms.

Replication-disabled RVs were one of the earliest virus vectors used for gene transfer in the brain and were used as a tool for the selective destruction of tumor cells. RVs in the brain seem to be well tolerated and biologically safe, although they are apparently unable to significantly extend the survival of brain tumor patients.

Data from the few brain tumor trials using HSV and AV are insufficient to draw reliable conclusions regarding the efficacy of these viruses, although safety seems to not be a critical issue in human patients. Although they have not provided the expected therapeutic breakthrough, clinical trials in gene therapy have nevertheless produced substantial amounts of data and have contributed to the identification of serious shortcomings and limitations of the strategy.

In the future, more attention needs to be paid to the integration of gene therapy strategies in combined multimodal protocols for brain tumors. Brain tumor gene therapy will certainly prove its potential as a potent and selective tumor-targeted adjuvant treatment option that is able to reduce morbidity and increase patients' quality of life while reasonably extending their life span.

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Current Gene Therapy, 2002, 2, 451-483

Neuroprotective Gene Therapy for Parkinson's Disease

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Abstract: Parkinson's disease (PD) is a neurodegenerative disease characterised by a progressive loss of the dopaminergic neurones in the substantia nigra pars compacta.

Accumulating evidence indicates that apoptosis contributes to neuronal cell death in PD patients' brain. Excitotoxicity, oxidative stress, and mitochondrial respiratory failure are thought to be the key inducers of the apoptotic cascade. Even though the initial cause and the mechanism of degeneration are poorly understood, neuroprotection can be achieved by interfering with neuronal cell death either directly or by preventing neuronal dysfunction. Potential agents for neuroprotection are neurotrophic factors, inhibitors of apoptosis or anti-oxidative agents. However, the existence of the blood-brain barrier precludes systemic delivery of these factors. *In situ* gene delivery provides strategies for local and sustained administration of protective factors at physiologically relevant doses.

Viral vectors mediating stable gene expression in the central nervous system exist and are still under development. Efficacy of these vectors has repeatedly been demonstrated in the animal models both *ex vivo* and *in vivo*. *Ex vivo* gene delivery could furthermore be combined with cell replacement therapies by transplanting genetically modified cells compensating for the lost neuronal cell population in order to provide neuroprotection to both the grafted cells and degenerating host neurones.

However, several aspects of gene transfer, such as uncontrolled diffusion, axonal transport, unpredictable site of integration and immunological responses, still raise safety concerns and justify further development of viral and non-viral vectors as well as genetic elements with tightly controlled gene expression.

Various relevant animal models for Parkinson's disease are available for the evaluation of gene therapy strategies. These include induction of cell death in specific neurone population through administration of toxins either directly in the brain or systemically, as well as transgenic mice expressing human disease-associated mutations.

1. INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease clinically characterised by resting tremors, rigidity and slowness of voluntary movements. Pathologic features of PD principally include a loss of the dopaminergic (DA) neurones in the mesencephalic substantia nigra pars compacta (SNpc) that massively projects to the striatum. This results in a severe depletion of striatal dopamine levels, mainly responsible for the motor symptoms associated with the disease [Lee *et al.* 1994] (see Fig. 1).

The aetiology of PD is still unclear (review:[Blum *et al.* 2001a]). Genetic predisposition, environmental toxins and the normal ageing have been suggested to be the contributing factors [Tanner 1992a;Tanner 1992b;Tanner *et al.* 1999]. However, recent studies suggest that mutations could be involved although they are generally observed in rare and early-onset cases. In particular, at least 2 missense mutations in the alpha-synuclein (α-SN) gene on chromosome 4q21-q23 have been identified in some Italian-American and Greek families and in one German family (Polymeropoulos *et al.* 1996; Polymeropoulos *et al.* 1997). The α-SN was identified as the major constituent of intranuclear neuronal inclusions called Lewy bodies, which are characteristic of PD [Spillantini *et al.* 1997]. Mutant forms of the soluble presynaptic α-SN have been shown to form oligomeres more readily [Conway *et al.* 2000b; Conway *et al.* 2000a; Conway *et al.* 1998] but whether these mutations can actually cause cell death is still ill-defined. In addition, mutations in the *parkin* gene, encoding a ubiquitin-like protein also present in Lewy bodies [Shimura *et al.* 2001], cause autosomal recessive juvenile parkinsonism.

In DA neurones of healthy individuals, dietary tyrosine is converted by tyrosine hydroxylase (TH) into L-DOPA, which is in turn converted into dopamine by aromatic amino acid decarboxylase (AADC). TH is the limiting enzyme in this process [Zigmond 1998]. Current pharmacological treatments for PD are based on the administration of dopamine receptor agonists in the early stages and adding the dopamine precursor, L-DOPA in later stages of the disease. L-DOPA is taken up by remaining dopaminergic

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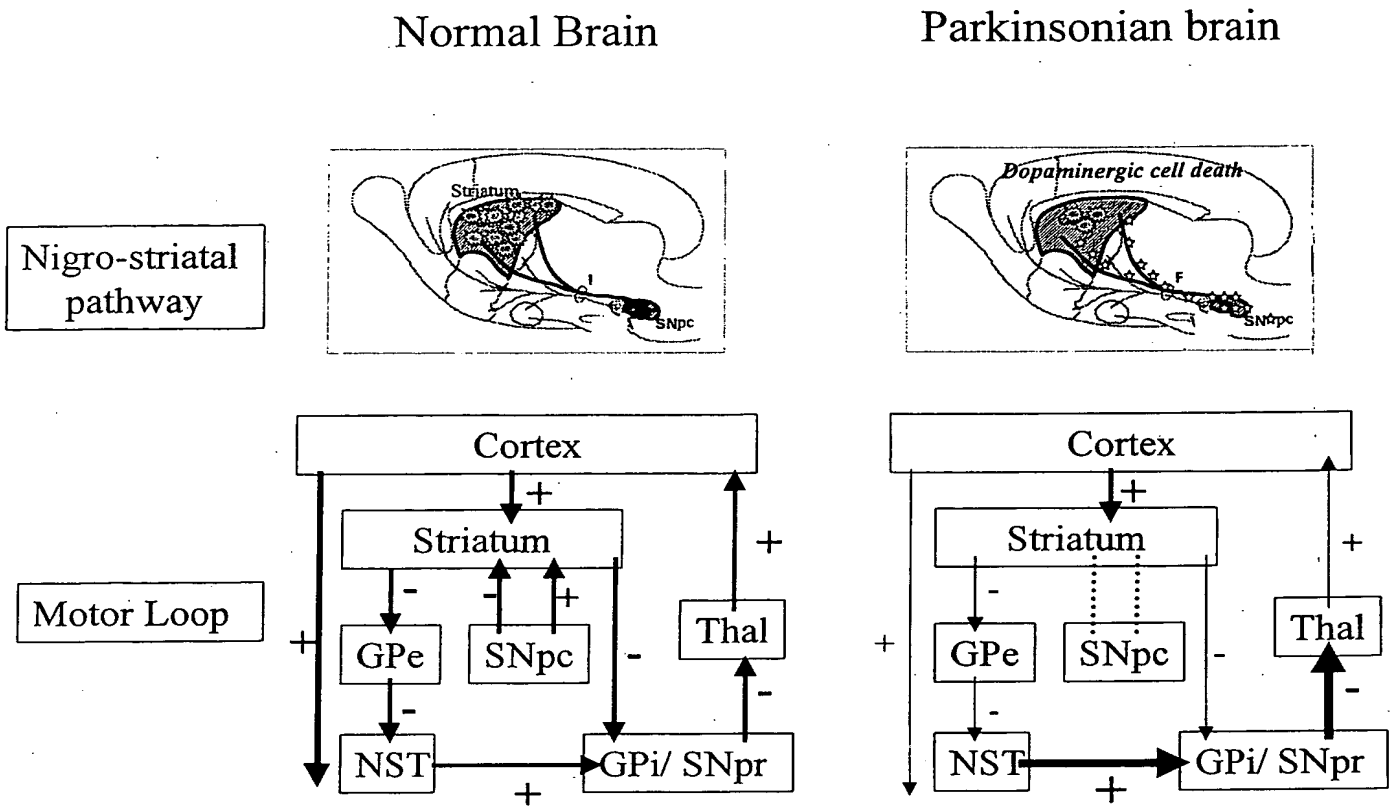

Fig. (1). The motor loop.

In the basal ganglia of the normal individual (on the left), the cortex sends activating projections to the striatum which in turn inhibit the internal globus pallidus (GPi) and the substantia nigra pars reticulata (SNpr) by either the direct or the indirect -via the subthalamic nucleus (NST)- pathways. GPi and SNpr inhibit the thalamus (Thal) which activates the cortex. SNpc regulates this loop by sending projections to the striatum which act on the dopamine receptors D1 (activating effect) and D2 (inhibitory effect).

In Parkinson's disease (on the right), the loss of control of the direct and indirect pathways by dopaminergic neurons of SNpc results in a net increase of the efferent inhibition of the Thal and hence of the thalamo-cortical activity and of the movement.

neurones and converted into dopamine, which is then released by the dopaminergic terminals in the striatum [Zigmond *et al.* 1990]. However, when the disease advances and the number of surviving dopaminergic neurones decreases, higher doses of L-DOPA have to be administered more frequently. This results in appearance of severe side-effects, mainly due to; i) high fluctuations of striatal doses of dopamine resulting in motor disturbances and ii) stimulation of extrastriatal dopamine receptors resulting in psychiatric symptoms.

Cell replacement therapy by heterotopic grafting of fetal DA neurones delivering dopamine directly and continuously in the striatum has proven clinically promising [Freed *et al.* 1992; Lindvall *et al.* 1992; Peschanski *et al.* 1994; Kordower *et al.* 1998; Levivier *et al.* 1997]. However, a wide applicability of this type of therapy is limited by the low availability and limited posttransplantation survival of the fetal tissue as well as by controversy regarding ethical issues. A recent clinical trial suggests that the protocol for tissue preparation, the amount and age of tissue transplanted as well as the location of the graft are crucial factors to be evaluated when moving from the laboratory to clinics [Freed *et al.* 2001].

The precise knowledge of the motor loop involving dopamine furthermore, has led to the development of compensatory therapies at the circuitry point of view, such as electrical stimulation of the globus pallidus or the subthalamic nucleus (structures that are part of the motor loop; see Fig. 1). Deep brain stimulation, when successful, drastically ameliorates the principal symptoms of the disease [Limousin *et al.* 1998]

Altogether, the relative success of specific therapies such as administration of a single neurotransmitter or local transplantation of neurotransmitter producing cells, suggests that among neurodegenerative diseases (NDD), PD is currently the most amenable to gene therapy. Indeed, given that cellular dysfunction is restricted almost exclusively to a single class of neurones in a well-defined location and results in the lack of a single neurotransmitter, local delivery

of a single or a restricted number of genes is envisageable. Therefore many new strategies were first tested on PD. However, non-treatable NDD, such as Huntington Disease or Amyotrophic Lateral Sclerosis, also constitute excellent challenges for new treatments such as those based on cell replacement and gene transfer, because even a little benefit is of great importance [Bachoud-Levi *et al.* 2000].

The first gene therapy-based strategy aimed at supplying locally, in a continuous and physiological manner, enzymes involved in the conversion of tyrosine into dopamine, such as TH, AADC as well as tetrahydrobiopterin (BH4), a co-factor necessary for TH activity [Jiao *et al.* 1996; Kaplitt *et al.* 1994a; During *et al.* 1998]. This strategy was first evaluated in a PD animal model consisting of an acute and specific lesioning of the nigrostriatal pathway with 6-hydroxydopamine (see section 3.1). If this strategy is to be applied in clinical therapy, presumably, the ongoing degeneration of dopaminergic neurones will progressively narrow the therapeutic window of gene expression levels. Therefore, as it is the case with L-DOPA administration, careful dosage of these enzymes and co-factors acting in the pathway of dopamine synthesis will be required. This in turn requires the use of precisely regulated promoters for gene expression, which are still under development (see section 7).

However, none of the treatments mentioned above (whether clinically available or experimental) address the progression of the disease and therefore eventually will cease to be effective in the advanced stages of PD. One goal is thus to interfere with the progress of the disease by halting or even reversing cell death and/or dysfunction.

Since neuronal degeneration in PD is slow and progressive, early diagnosis when substantial numbers of neurones are still alive could pave the way for effective neuroprotective therapies. Indeed, neuroprotection will only be effective when there are cells still alive and when reinnervation is still possible. In the later stages of the disease, only compensatory treatments might be effective. This is a major problem for researchers and clinicians since at the time the disease is diagnosed already more than 50% of the dopaminergic neurones have already died [Agid and Blin 1987].

The goal of neuroprotective gene therapy (NPGT) for PD is to prevent further degeneration of dopaminergic neurones. To achieve this, the mechanisms and cause(s) of cell death should be characterized. Therefore, fundamental studies of neuronal cell death in diseased brain are of overwhelming importance. If the mechanism of cell death is apoptotic (section 2.2), delivering antiapoptotic genes in dopaminergic neurones might be neuroprotective. The signal transduction and further apoptotic genes cascade should be studied in the particular case of PD, in order to define targets to efficiently interfere with these processes. The identification of the agent(s) that induce cell death, provided they are causative, is also likely to provide strategies that prevent the cells to even enter the process of cell death. If cell death is triggered by oxidative stress (see section 2.1), genes involved in the detoxification of free radicals such as superoxide dismutase (SOD) or catalase might be effective.

After the therapeutic gene has been chosen, several features have to be controlled:

i) The target cell population. When an autocrine or intracrine effect on dopaminergic neurones is expected, a vector that is able to transduce dopaminergic neurones should be used. In contrast, when a paracrine effect is expected, the vector could be delivered either in the striatum if the gene product is expected to protect the dopaminergic terminals or in the SNpc when it is expected to protect the dopaminergic cell bodies. The pathogical situation may influence the choice of a strategy. For example, rescuing degenerating cells in the SNpc might be useless when the innervation in the striatum has already disappeared, since reinnervation at distance usually does not occur in the adult (see discussion).

ii) The kinetics and level of gene expression. PD being a progressive disease, neuroprotective agents have to be administered for very long periods in relevant amounts in order to counteract the disease. Vectors able to achieve sustained gene expression are thus required. However, excess of particular neurotransmitters as well as disturbance of the intricate regulatory loops by exogenous intervention is likely to cause severe adverse effects. The level and durability of gene expression need thus to be adjusted in order to minimise undesirable effects (see section 7). Motor disturbances have been described resulting from excess of compensatory cells in animals [Schierle *et al.* 1999], inappropriate age or location of the grafted tissue in humans [Freed *et al.* 2001], as well as inappropriate expression of genes encoding neurotrophic factors in animal models [Kirik *et al.* 2000a].

Theoretically, transferred DNA sequences cannot be recovered after being introduced in the patients, except in the particular case of *ex vivo* gene therapy using encapsulated genetically-modified cells [Aebischer *et al.* 1996a], where capsules may be removed. However, the activity of the gene construct can be controlled by the use of regulatory elements ensuring a transcriptional control of gene expression (see [Smith-Arica *et al.* 2000] and references therein). Grafted genetically-modified cells can be eliminated by incorporating suicide genes in the vectors and administering the prodrug if the termination of the treatment is required [Aebischer *et al.* 1996b].

Further development and/or characterization of vectors for gene delivery is urgently needed in order to meet these requirements.

The development of relevant animal models is also a limiting step in establishing clinically relevant protocols for neuroprotective gene therapy. Indeed, even if models faithfully mimic clinical symptoms and histopathological findings, the possibility that the mechanism of induction of experimental PD does not reflect the situation occurring in human disease still exist. Therefore, converging data from several models induced by different triggers (e.g. acute toxicity, chronic toxicity, mutation, etc.) should be obtained before clinical trials can be initiated (see section 3).

2. CELL DEATH IN PARKINSON'S DISEASE

2.1. Oxidative Stress and Mitochondrial Deficiency

The brain consumes large amounts of oxygen and is thus particularly prone to oxidative damage. There is significant evidence, based on post-mortem analysis as well as animal and cellular models, that the generation of reactive oxygen species (ROS) and mitochondrial dysfunction are involved in nerve cell death in Parkinson's disease [Beal, 1995]. Particularly, chemical oxidation of dopamine produces potentially harmful semiquinones and metabolism of dopamine by monoamine oxidase results in formation of hydrogen peroxide which, in the presence of iron is converted into highly reactive hydroxyl radicals. In healthy brain, there is a balance between ROS generation and antioxidative systems. The major antioxidant defenses consist of scavengers such as glutathione, vitamin C or vitamin E, and antioxidant enzymes, including Cu, Zn and manganese superoxide dismutase (SOD), which convert 0_2• to H_2O_2 and catalase and selenoglutathione (GSH) peroxidase, which catalyze the convertion of H_2O_2 to H_2O.

Oxidative stress describes a condition in which cellular antioxidant defenses are insufficient to keep the levels of ROS below a toxic threshold. This may either be due to excessive production of ROS or the loss of antioxidant defenses.

Recent evidence obtained on patients' brain suggest that oxidative stress plays an important role in PD [Beal 1995]. These include a selective decrease in mitochondrial complex I activity [Janetzky *et al.* 1994; Schapira *et al.* 1990; Hattori *et al.* 1991] as well as reduced glutathione levels in the SNpc [Sian *et al.* 1994b; Sian *et al.* 1994a; Sofic *et al.* 1992], mutations in mitochondrial DNA [Mecocci *et al.* 1993] and ROS-mediated DNA damage [Spencer *et al.* 1994]. Excessive production of hydroxyl radicals in the SNpc of parkinsonian patients is further suggested by the fact that iron concentrations in SN are 70% higher as compared with healthy individuals. Oxidative damage to mitochondrial DNA shows age-dependent increase in human brain [Mecocci *et al.* 1993]. On the other hand, an age-related decline in GSH peroxidase activity [Sohal and Weindruch 1996] as well as in mitochondrial electron transport [Bowling *et al.* 1993;Bowling and Beal 1995] has also been observed.

Consistently, SOD-deficient mice show premature neurodegeneration and death [Lebovitz *et al.* 1996]. Catalase and GSH peroxidase are complementary to detoxify H_20_2. Inhibitors of either enzyme only marginally reduce the rate of disappearance of H_20_2. However, inhibition of both enzymes strongly reduces H_20_2 clearance [Dringen and Hamprecht 1997]. In pathological conditions, however, when the amount of ROS is particularly high, either of these enzymes becomes limiting. For example, mice deficient in GSH show increased vulnerability to 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), an inhibitor of mitochondrial complex I activity, used to produce a model of PD (see section 3). The observation of decreased cell death in transgenic mice overexpressing SOD and GSH peroxidase further supports the role of ROS in MPTP-mediated neuronal cell death [Asanuma *et al.* 1998;Bensadoun *et al.* 1998]

Excitotoxicity, another possible contributor for oxidative stress involved in PD, refers to neuronal cell death caused by overactivation of excitatory amino acid receptors, especially under metabolic and oxidative stress. An inability to maintain cellular ATP levels may lead to partial neuronal depolarization and persistent receptor activation by ambient glutamate levels [Novelli and Tasker 2000]. Indeed, glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when cellular energy levels are reduced. Low cellular energy also interferes with calcium buffering. Increased intracellular calcium levels can initiate a number of deleterious processes including activation of nitric oxide synthetase (NOS) and generation of nitric oxide, which is thought to be an important mediator of neuronal damage in PD [Hantraye *et al.* 1996]. Thus, oxidative stress may promote excitotoxic mechanisms and altered energy metabolism leading to disturbed interneuronal calcium homeostasis and apoptosis.

2.2. Apoptosis in PD

Increasing evidence indicates that apoptosis contributes to neuronal cell death in PD. Apoptosis was originally described as a physiological mode of cell death. Cells undergoing apoptosis are characterized by several morphological changes such as chromatin condensation, blebbing of plasma membrane, cell shrinkage and appearance of apoptotic bodies. [Kerr *et al.* 1972; Clarke 1990].

Evidence for an apoptotic mechanism of cell death in post-mortem human PD brain has been obtained using the TUNEL method that allows to demonstrate *in situ* DNA fragmentation, a characteristic feature of the final step of apoptosis [Dragunow *et al.* 1995; Thomas *et al.* 1995; Mochizuki *et al.* 1996]. Other studies, demonstrated the presence of apoptotic cells by morphological tools [Arglade *et al.* 1997; Tatton *et al.* 1998]. However, some authors, failed to reproduce these results [Banati *et al.* 1998;Jellinger 2000]. The reasons for these discrepancies are unclear (for discussion see [Blum *et al.* 2001a]). This could either be due to the lack of specificity of the TUNEL method regarding the detection of apoptosis [Charriaut-Marlangue and Ben Ari 1995], the agonal conditions of the patients or to the methods used for tissue fixation [Kingsbury *et al.* 1998]. Furthermore, given the slow progression of PD (over several years) and the rapidity of the apoptotic process (few hours), a very low percentage of cells are expected to undergo cell death at the time of analysis.

However, several recent works suggested that neuronal cell death occurring in PD may be gene-regulated, favoring the apoptotic hypothesis. Indeed, modulations of the expression of pro-apoptotic or anti-apoptotic proteins of the Bcl-2 family such as Bax or Bcl-2 itself have been described [Tatton 2000;Marshall *et al.* 1997; Mogi *et al.* 1996]. Other studies suggest an activation of various caspases, responsible for the execution phase of apoptosis [Tatton 2000]. Futhermore, constitutive presence of caspases as inactive

pro-enzyme in the neuronal cells could constitute a significant vulnerability factor to degeneration [Hartmann *et al.* 2000; Hartmann *et al.* 2001]. Transcription factors such as p53 and NF-κB, known to be intimately linked to the cell death process (for review see [Barkett and Gilmore 1999] were also shown to be activated in PD brain [de la Monte *et al.* 1998; Hunot *et al.* 1997].

At present, given the lack of etiological clues for PD, the nature of the injuries which induce apoptosis is unknown. However, numerous phenomena-oxidative stress, respiratory chain defects, cytokines release (i.e. TNFα), paucity of neurotrophic factors, mutated α-SN, exogenous/endogenous toxic factors or neuronal excitotoxicity-known to experimentally induce apoptosis and to be physiopathologically relevant in PD could be, at least partly, responsible for apoptosis (for review see [Mattson *et al.* 2000; Hajimohamadreza and Treherne 1997; Blum *et al.* 2001a; El Agnaf *et al.* 1998]).

Therefore, the overwhelming biochemical and molecular defects characterized in PD converge to apoptosis. However, at present, it appears difficult to evaluate the potential therapeutic significance of neuroprotective anti-apoptotic strategies based on the gene-mediated expression of anti-apoptotic factors (Bcl-2-like proteins, caspases inhibitors or IAP). Indeed, it remains clear that merely acting on the molecular events described above will not be sufficient to restore normal neuronal function because they reflect irreversible dysfunctions.

3. MODELS

Various animal models mimicking PD have been developed. Dopaminergic cell death can either be induced: i) by axotomy of the nigrostriatal nerve fibers; ii) by administration of toxins either directly or systemically; or iii) by overexpression of human disease related genes or their mutated forms. Ultimately the relevance of these models can only be evaluated by comparing the histopathology and symptoms induced with those observed in humans.

3.1. 6-Hydroxydopamine (For a Review: [Gerlach and Riederer 1996; Blum et al., 2001]; see Fig. 2)

A widely used model for PD consists in the injection of the catecholamine-specific neurotoxin 6-hydroxydopamine (6-OHDA) in the SNpc or in the medial forebrain bundle

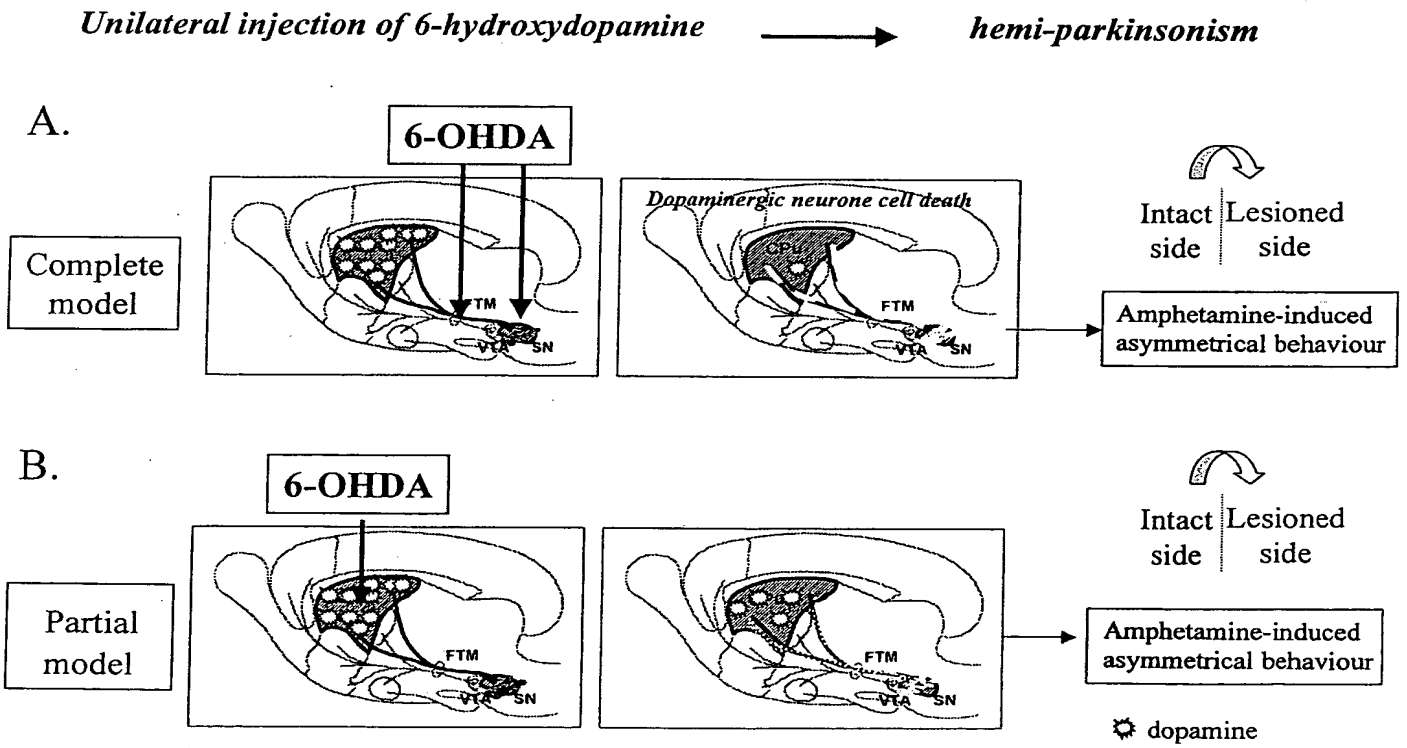

Fig. (2). The 6-hydroxydopamine models for Parkinson's disease.

Unilateral injection of 6-hydroxydopamine, a toxin which specifically destroys catecholinergic neurones induces an unilateral dopaminergic denervation of the striatum called hemiparkinsonism. Functionnal evaluation of the lesion is performed by intraperitoneal injection of amphetamine, a drug which stimulates dopamine release in the intact striatum, thus inducing a rotational behaviour towards the lesioned side.

A. The complete model, which mimics the late stages of PD, consists in the destruction of more than 90% of the dopaminergic neurones by injecting 6-OHDA either directly in the SNpc or in the medial forebrain bundle (the dopaminergic fibers which project to the striatum).

B. The partial model, which mimics early stages PD consists in the injection of 6-OHDA in the striatum, thus provoking a partial retrograde degenerescence of SNpc dopaminergic neurones.

(which contains the nigro-striatal projections) of rats. This produces a destruction of nigral DA neurones as well as a striatal depletion in dopamine which are the main physiopathological features responsible for motor impairments in PD [Ungerstedt and Arbuthnott 1970]. 6-OHDA-induced SNpc degeneration produces an asymmetric and quantifiable motor behavior after unilateral lesion followed by systemic administration of either dopamine receptor agonists (e.g. apomorphine) or dopamine releasing drugs (Hefti *et al.* 1980). This allows an easy and reliable control of the extent of the lesion and an evaluation of the potential benefit of therapeutic treatments. However, this model consists in an acute injury of the nigrostriatal system and does not mimic the progressive and long-term degeneration observed in PD. Furthermore, it results in a nearly complete destruction of DA neurones and hence mimics the "end-stage" of the disease, which is of limited interest for the evaluation of neuroprotective approaches. The end-stage model is nevertheless very useful to study substitutive therapies such as transplantation of fetal DA neurones or gene therapy strategies consisting in providing enzymes that compensate the lack of dopaminergic terminals in the striatum [Mandel *et al.* 1999a].

A partial degeneration of DA neurones can be obtained by injection of a limited amount of 6-OHDA in the lateral part of the SNpc resulting in the destruction of ~50% of the dopaminergic nigral cell population (Agid *et al.* 1973). A partial and progressive lesioning of the dopaminergic system over several weeks can also be produced by injecting 6-OHDA in the striatum [Sauer and Oertel 1994; Przedborski *et al.* 1995]. This induces retrograde partial degeneration. However, the lesion reaches very rapidly to a static situation.

As observed in PD patients, oxidative stress and apoptosis are induced in dopaminergic neurons of 6-OHDA-treated animals [He *et al.* 2000;Marti *et al.* 1997; Burke and Kholodilov 1998]as well as in culture [Blum *et al.* 1997;Walkinshaw and Waters 1994].

3.2. 1 - Methyl - 4 - Phenyl - 1, 2, 3, 6 - Tetrahydropyridine (MPTP) (For a Review: [Przedborski et al. 2001])

In the late seventies, a population of young Californian people addicted to new synthetic heroin was shown to have developed an irreversible L-DOPA responsive PD [Davis *et al.* 1979]. Analysis of this synthetic drug showed that it contained about 3 % of MPTP [Langston *et al.* 1983]. Drug users exhibited symptoms very similar to what is seen with PD including development of bradykinesia, rigidity, postural instability and resting tremors. Unlike in PD, the most active phase of degeneration following MPTP administration is completed within a few days [Langston 1987]. However, it has been suggested that following the early phase of neuronal death, MPTP-induced neurodegeneration may continue to progress over several decades [Vingerhoets *et al.* 1994; Langston *et al.* 1999]. Postmortem investigations clearly confirmed the lesioning of the SNpc [Davis *et al.* 1979]. However, Lewy bodies, characteristic of PD have not been convincingly observed in MPTP-induced Parkinsonism [Forno *et al.* 1993].

MPTP is a powerful tool to induce nigral degeneration in animals as well as PD-like pathology in several species such as rat, mouse, dog, cat, and monkey. When administrated to animals, MPTP rapidly crosses the blood-brain barrier and is converted, mainly in glial cells, into its effective form namely 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B. MPP^+ then accumulates into dopaminergic cells after selective uptake by dopamine uptake sites. MPP^+ enters mitochondria by an energy-dependent mechanism [Ramsay *et al.* 1986] and inhibits the activity of this organelle, leading to a drop in cellular ATP levels and subsequent cell death. The effects of MPTP on animals depend on several parameters such as administration mode, dosage and animal age [Gerlach and Riederer 1996; Tipton and Singer 1993]. Interestingly, studies on mice support the notion that older animals are more susceptible to MPTP [Jarvis and Wagner 1985].

Involvement of ROS has been suggested by the cellular protection provided by increasing SOD expression in transgenic mice [Przedborski *et al.* 1992] and the potentiation of MPTP effects by either deficiency in SOD or GSH peroxidase genes [Klivenyi *et al.* 2000;Storch *et al.* 2000;Zhang *et al.* 2000]. Apoptosis was observed in the SNpc of mice treated with MPTP [Spooren *et al.* 1998; Tatton and Kish 1997]. The mechanism of MPP^+-induced cell death has also been studied in neuronal cell cultures [Storch *et al.* 2000].

Both the acute and chronic models of MPTP-induced parkinsonism in monkey exist. In the acute model, monkeys are treated with a high dose of MPTP [Irwin *et al.* 1990], whereas in the chronic model low doses of MPTP are administered for a prolonged period of time in an attempt to achieve progressive PD [Bezard *et al.* 1997]. In this model of PD, MPTP induces the loss of pigmented neurones of the SNpc [Hantraye *et al.* 1993]. Interestingly, the topology of the nigral lesion after MPTP treatment is similar to that observed in human PD since pars lateralis of the SNpc appeared more affected than medial part [Varastet *et al.* 1994]. Presumably, the chronic model is the most suitable for testing neuroprotective strategies.

3.3. Transgenic Animals

The identification of genetic components in PD has recently led to the development of transgenic animal models and opened perspectives for new (gene) therapeutic strategies. Ablation of the gene encoding α-synuclein (α-SN) in mice results in functional deficits of the nigrostriatal dopamine system [Abeliovich *et al.* 2000] providing further evidence for a connection between α-SN and parkinsonism. Several α-SN transgenic mice have now been reported overexpressing the wild-type, A30P, A53T or double mutant form of the protein under control of different promoters [Giasson *et al.* 2002; Kahle *et al.* 2000; Lee *et al.* 2002; Masliah *et al.* 2000; Matsuoka *et al.* 2001; Rathke-Hartlieb *et al.* 2001; Richfield *et al.* 2002; van Der *et al.* 2000]. In almost all of these animals, overexpression of α-SN induces abnormal cytoplasmic and neuritic inclusions of the protein [Kahle *et al.* 2000], that are sometimes ubiquitinated [Giasson *et al.* 2002; Lee *et al.* 2002; Masliah *et al.* 2000;van

Der *et al.* 2000]. Alpha-SN fibrils in these inclusions were only reported with the prion protein promoter [Giasson *et al.* 2002; Lee *et al.* 2002], or in combination with overexpression of human β-amyloid peptide [Masliah *et al.* 2000]. Some of the transgenic animals also display mild to severe motor impairments [Giasson *et al.* 2002;Masliah *et al.* 2000]. However, a clear correlation with dysfunction of the dopaminergic system could not be established. Loss of dopaminergic terminals is found occasionally [Masliah *et al.* 2000; Richfield *et al.* 2002] but no loss of dopaminergic neurons was reported, not even when α-SN expression is under control of the tyrosine hydroxylase promoter .

Overexpression of wild-type and mutant α-SN in drosophila model of PD on the other hand strikingly reproduces many features of PD [Feany and Bender 2000]. There is an age-dependent specific loss of dopamine-secreting neurones starting at mid-life. Some neurones accumulate intracellular aggregates containing α-SN fibrils that closely resemble Lewy bodies. Moreover, the flies display a progressive, age-dependent loss of motor function.

Although the fruit fly model resembles PD at first sight and enables genetic studies, it still seems that drug screening and gene therapy would be more relevant in rodents. In neuronal cell cultures a link between α-SN expression, oxidative stress [Hsu *et al.* 2000;Ko *et al.* 2000] and induction of apoptosis [Saha *et al.* 2000] has been shown. The absence of dopaminergic cell loss in α-SN transgenic mice is therefore puzzling. In conclusion, these first reports on α-SN transgenic animals not only confirm the importance of α-SN in PD but also stress the need for even better experimental animal models that will allow further research on the pathogenesis of the disease and on the new therapeutic targets and strategies.

3.4. Local Transgenesis: Viral-Mediated Genetic Models

In addition to the therapeutic potential, stereotaxic viral vector-mediated gene transfer provides a new method to create disease models by inducing acute, stable, locoregional overexpression of disease-associated genes in the adult brain.

Parkinson-like neurodegeneration recapitulating the main features of the disease could be generated using recombinant virus-mediated transfer of the wild-type or mutant form of the human α-SN gene locally in the substantia nigra [Klein *et al.* 2002; Lo *et al.* 2002;Kirik *et al.* 2002; Lauwers *et al.*, submitted[1]]. In these studies, accumulation of granular α-SN in cytoplasmic inclusions, neuritic dilatations, decreases of striatal dopamine levels and progressive neurodegeneration (up to 80%) of α-SN positive cells was observed. However, the α-SN inclusions seem to lack the classical fibrillar structures. In some of the animals, behavioral deficits could be correlated with dopaminergic cell death [Kirik *et al.* 2002]. Altogether, local overexpression of α-SN mediated by viral vectors, results in more pronounced cell death and pathological changes that start relatively early (from 6 weeks after injection on) as compared to the reported transgenic mice. This might be explained by the fact that expression of the transgene is initiated at adult age, making compensatory changes in gene expression or induction of alternative pathways to counteract the transgene phenotype less likely to occur.

4. NEUROPROTECTIVE STRATEGIES

Neuroprotection can be envisaged at different levels:

- before the process leading to cell death is turned on, e.g. by scavenging the cell death inducing agents (such as, SOD, catalase,etc,) or by rendering the cells more resistant to stress (e.g. neurotrophic factors);

- when the process is already engaged, by interfering in signal transduction at the level of apoptotic and anti-apoptotic genes (e.g. bcl-2), execution genes (e.g. caspases), etc..

4.1. Neurotrophic Factors

Neurotrophic factors (NF) generally regulate neurone survival and differentiation and protect them against various insults [Lewin and Barde 1996]. The prototypical NF is "nerve growth factor" (NGF), originally identified by its ability to protect peripheral sensory and sympathetic neurones from programmed cell death during development [Levi-Montalcini 1982] as well as against a variety of insults such as axotomy, hydrogen peroxide, excitatory amino acids, etc. Notably, NGF was shown to induce expression of SOD and catalase [Goins *et al.* 1999]. NGF also protects cultured dopaminergic neurones against MPP^+ toxicity [Shimoke and Chiba 2001].

Brain-derived neurotrophic factor (BDNF) gene shares significant homology with the NGF gene. Like NGF, it binds with high affinity to specific members of the tropomyosin-related kinase (Trk) family of receptors [Squinto *et al.* 1991; Soppet *et al.* 1991]. Like NGF, it promotes the survival and differentiation of dopaminergic neurones *in vitro* [Hyman *et al.* 1991;Spina *et al.* 1992; Spenger *et al.* 1995] and protects them against MPP^+ and 6-OHDA-induced toxicity [Hyman *et al.* 1991;Spina *et al.* 1992].

In vivo BDNF increases dopaminergic metabolism in healthy as well as 6-OHDA lesioned animals [Altar *et al.* 1992; Altar *et al.* 1992]. However, several studies failed to show that BDNF has neuroprotective effects in other animal models of PD. Indeed, intranigral injection of BDNF did not protect against medial forebrain bundle axotomy [Lapchak *et al.* 1993] or intranigral injection of 6-OHDA [Altar *et al.* 1992]. In contrast, BDNF gene delivered by cellular [Frim *et al.* 1994; Levivier *et al.* 1995a] and viral vectors [Klein *et al.* 1999] protect dopaminergic neurones in partial, but not in complete 6-OHDA model (see sections 5.1 and 5.2.3).

Neurotrophins including NGF, BDNF, neurotrophins (NT)-3, -4 and -5 activate receptors of two different classes: the Trk family of receptor tyrosine kinases and the p75

[1] Lauwers, E., Debyser, Z., Van Dorpe, J., De Strooper, B., Nuttin, B. and Baekelandt, V. Neuropathology and neurodegeneration in rodent brain induced by lentiviral vector-mediated overexpression of α-synuclein. submitted.

receptor, a member of the tumor necrosis factor (TNF) receptor family [Patapoutian and Reichardt 2001]. Trk receptors seem to mediate almost all of the survival promoting effects of neurotrophins with NGF signaling through TrkA, BDNF and NT-4/5 through TrkB, and NT-3 through TrkC.

The most potent neurotrophic factor for dopaminergic neurones identified until now is the "glial cell line-derived neurotrophic factor" (GDNF), a member of the transforming growth factor-β superfamily [Lin *et al.* 1993].

In vitro, GDNF promotes survival of cultured DA neurones [Lin *et al.* 1993; Hou *et al.* 1996] and stimulates their morphological differentiation in terms of increased neurite outgrowth [Costantini and Isacson 2000] and soma size [Lin *et al.* 1993]. Notably, it is not mitogenic [Lin *et al.* 1993].

Protective effects of GDNF have been demonstrated *in vivo* (see Fig. 3). Administration of recombinant GDNF into the SNpc of rats and monkeys prior to injection of toxins protects the nigrostriatal pathway [Kearns and Gash 1995;Sauer *et al.* 1995;Tomac *et al.* 1995a]. Restorative effects have also been demonstrated. Administration of GDNF after removal of MPTP prevents further cell death and stimulates regrowth of fibers [Tomac *et al.* 1995a]. Furthermore, stabilized 6-OHDA lesions can still be partially reversed by GDNF administration as the TH^- "ghost" cells regain their phenotype. GDNF can also enhance the functioning of dopaminergic neurones in aged animals [Fox *et al.* 2001]. However, although intranigral injections of GDNF exert protective and reparative effects in the SNpc, they have no effect on striatal dopamine levels [Rosenblad *et al.* 2000a]. In contrast, when injected in striatum of MPTP-treated mouse [Tomac *et al.* 1995b] or 6-OHDA-treated rats [Rosenblad *et al.* 2000a], GDNF increased dopamine levels both in the SNpc and in the striatum. The fact that GDNF can be retrogradely transported from the striatum to the SNpc [Tomac *et al.* 1995b] provide a clue for the latter results. However, injection of large doses of recombinant GDNF protein causes adverse effects such as increased locomotor activity and loss of body weight [Hudson *et al.*

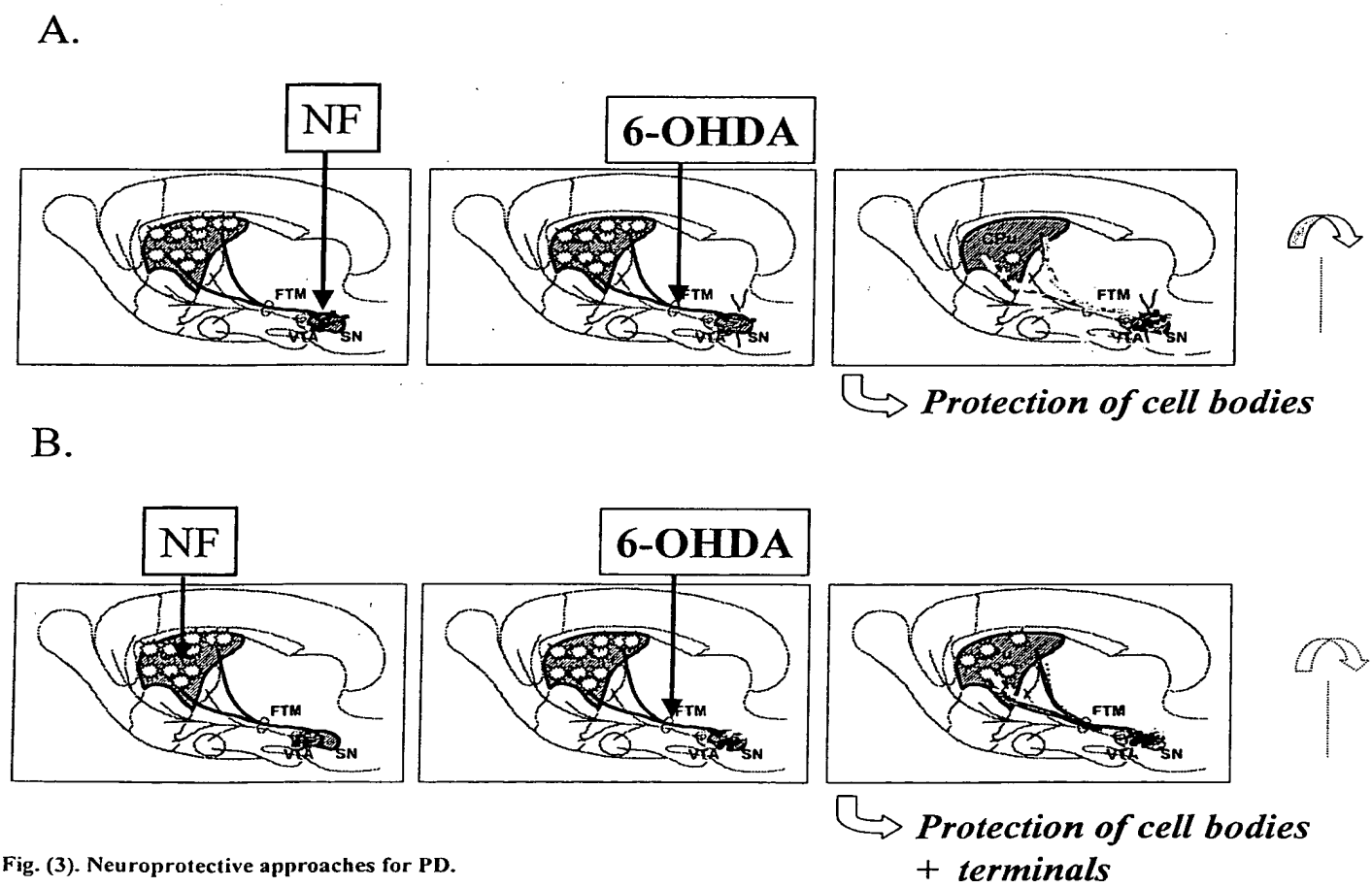

Fig. (3). Neuroprotective approaches for PD.

Neurotrophic factors, such as GDNF can protect dopaminergic neurons against injury.

A. When injected in the SNpc prior to administration of 6-0HDA, NF efficiently protect dopaminergic cell bodies but striatal innervation is lost. Consequently, there is no behavioural recovery.

B. When injected in the striatum, NF protect both dopaminergic cell bodies in SNpc and dopaminergic terminals in the striatum. Consequently, no motor symptoms appear.

1995; Martin *et al.* 1996a;Martin *et al.* 1996b].

The precise mechanism of GDNF action is not known but it requires a presence of GPI-linked proteins (GFR-α), which bind GDNF with high efficiency. GDNF-GFR-α forms a complex with the membrane-associated receptor Ret, thereby inducing receptor tyrosine kinase autophosphorylation and activation. Many of the surviving dopaminergic neurones from PD brains still express Ret [Walker *et al.* 1998], supporting the relevance of using GDNF as a potential therapeutic agent.

Other NF stimulating survival of mesencephalic DA neurones include the neurotrophins NT4/NT5 [Hynes *et al.* 1994]; basic fibroblast growth factor (bFGF ; [Beck *et al.* 1993]); epidermal growth factor (EGF; [Ferrari *et al.* 1989]); insulin-like growth factor (IGFs; [Knusel *et al.* 1990]);and transforming growth factor α (TGFα; [Widmer *et al.* 1993; Widmer *et al.* 1993]). NT4/5 was also found to exert neurotrophic effects *in vivo* [Haque *et al.* 1996].

More recently, Neurturin (NTN), another member of the GDNF family has been cloned [Kotzbauer *et al.* 1996]. Like GDNF, it is expressed in the developing and adult nigrostriatal system and promotes survival of fetal dopaminergic neurones [Horger *et al.* 1998]. It was also shown to signal through the GFR-α/Ret pathway [Cacalano *et al.* 1998]. In the partial 6-0HDA model, NTN has a similar potency as GDNF when injected at the level of the SN, but is less efficient when administered at the level of the striatum [Rosenblad *et al.* 1999]. This has tentatively been attributed to a poor diffusion of NTN in the brain.

Systemic administration of recombinant neurotrophic factors (NF) has been hampered by peripheral side effects and unability to cross the blood–brain barrier [Martin *et al.* 1996a; Miller *et al.* 1996]. Furthermore, given the short half-life of recombinant neurotrophic factors [Dittrich *et al.* 1994], high doses, which generally provoke adverse effects, have to be applied in a continuous manner. Therefore, local delivery of recombinant neurotrophic factors in the CNS is unfeasible.

Gene therapy provides strategies for continuous and sustained delivery of neurotrophic factors at the physiological doses, thus avoiding side-effects of high doses and contamination risks of continuous administration.

4.2. Overexpressing Enzymes Involved in the Elimination of Free Radicals

Based on the largely documented hypothesis that ROS are involved in the induction of neuronal cell death, antioxidants such as lazaroids have successfully been used to improve dopaminergic graft survival [Nakao *et al.* 1994]. Therefore, an alternative neuroprotective strategy could consist in reducing the amount of free radicals by raising the intracellular levels of enzymes that detoxify oxygen free radicals. Grafts of fetal ventral mesencephalon prepared from mice overexpressing Cu/Zn superoxide dismutase show an increased post-transplantation survival rate [Nakao *et al.* 1995].

4.3. Interfering with the Apoptotic Cascade (For a Review: see [Robertson et al. 2000])

Accumulating evidence suggests that transfer of anti-apoptotic genes could block the progression of neurodegeneration.

Dopamine-induced apoptosis of PC-12 cells is inhibited by overexpression of bcl-2 [Offen *et al.* 1997]. Cultured neurones from bcl-2 overexpressing transgenic mice are resistant to 6-0HDA toxicity *in vitro* [Offen *et al.* 1998]. Bcl-2 overexpressing mice were protected against MPTP toxicity [Yang *et al.* 1998].

The limitation of this approach for NPGT, as compared to overexpression of NF or detoxifying enzymes, is that since bcl-2 is acting intracellularly, only cells overexpressing the gene are expected to be protected. This approach thus requires the use of vectors able to transduce DA neurones with a very high efficiency (close to 100%).

5. GENE DELIVERY

Vectors for delivering neuroprotective genes include cellular vectors, viral vectors and non-viral vectors. The characteristics, advantages and drawbacks of each of them are discussed through examples.

5.1. Cellular Vectors

The first approaches for neurotrophin gene delivery in the models of neurodegenerative diseases were of using genetically modified heterologous cells secreting NF. To demonstrate the feasibility of the approach, fibroblasts, which can easily be grown and transduced *in vitro* with retroviruses, have been used. For example, implantation of BDNF-expressing fibroblasts into the striatum of rats protects them against the toxicity of a subsequent striatal injection of 6-OHDA [Levivier *et al.* 1995b].

Genetically modified fibroblasts have been used as a model system, which produced highly valuable scientific data regarding the effects of neuroprotective gene transfer. However, their inocuity as a cellular vector for clinical use has to be questioned. Indeed these cells secrete an uncontrollable variety of substances, which are likely to exert diverse paracrine effects on host cells. For example, transplantation of fibroblasts in the the rat striatum was itself shown to induce short-term neuroprotection [Levivier *et al.* 1995a]. Immune and inflammatory responses as well as tumor formation [Horellou *et al.* 1990] were also described in this model.

To avoid tumor formation, capsules made of a permselective membrane allowing the inward diffusion of nutrients and the outward diffusion of NF have been proposed [Sagot *et al.* 1995]. Nevertheless, transplanted encapsulated genetically modified cell lines, especially of nonneuronal origin, may secrete irrelevant substances, which could affect the host cells. For example, astrocytosis has been reported in some studies [Kordower *et al.* 1994; Hoffman *et al.* 1993].

Endothelial cells, such as brain microvascular endothelial cells, may be suitable cellular vectors for gene delivery to the CNS [Johnston *et al.* 1996]. They can undergo genetic modification, proliferate *in vitro* and survive transplantation. However, only short-term expression of the transgene after transplantation has been demonstrated.

Current trends are focused on neuronal precursors or stem cells that might provide an alternate source of cellular vectors that could be able to differentiate in response to epigenetic stimuli and to integrate in the environment of the host brain. They could provide ideal cellular vectors to deliver appropriate neuroprotective genes. The potential of the stem cells for combined cell replacement and gene therapies is discussed in section 6.

5.2. Viral Vectors

The use of retroviral vectors for gene therapy of the CNS is limited to *ex vivo* strategies since these vectors can only infect dividing cells. In contrast, lentiviral vectors, are able to transduce postmitotic cells (see section 5.2.4) and can be used for direct *in vivo* gene delivery. Vectors based on adeno-associated virus (AAV) and on lentiviruses, such as the human immunodeficiency virus (HIV), potentially present major advantages over adenoviral and herpes-based vectors: i) they might be less immunogenic; ii) they provide stable gene expression in non-dividing cells: HIV vectors integrate in the cellular genome whereas AAV vctors are maintained as stable episomes consisting of large concatemers of the viral genome and possibly integrate at long-term.

5.2.1. Vectors Based on Herpes Simplex Virus

Herpes simplex virus (HSV) is a large neurotrophic human virus containing 152 kb of linear double-stranded DNA. It can cause virulent lytic infection or stay in a lysogenic state in the CNS [Glorioso *et al.* 1994]. Episomal HSV genomes can become lytic after many years in a latent state. The neurotropism of this virus has prompted the development of vectors for gene delivery to the nervous system.

HSV-based vectors are deleted in essential genes to make them replication-defective. HSV vector stocks are prepared using complementing cell lines. They efficiently transduce neurones in culture in which they are maintained as episomes. *In vivo* they show a tropism for the nervous system in which both neurones and glia are transduced. However, relatively short-lived transgene expression has been obtained so far [Lachmann and Efstathiou 1999]. Loss of expression is not due to vector clearance as vector genomes persist in the brain for up to a year.

The main limitations of the herpes virus-based vectors are immune responses against viral proteins and cytotoxicity. Cytotoxicity appears to be mediated by immediate early genes. Indeed, UV irradiated virus is not toxic even at high multiplicity of infection.

The human bcl-2 cDNA under the control of a CMV promoter was cloned in an HSV vector, which was injected in SNpc one week prior to a 6-OHDA striatal lesion [Yamada *et al.* 1999]. Two weeks following the injection, approximately twice as many TH immunoreactive cells of the SN survived in the bcl-2 treated animals as compared to control vector (lacZ) treated animals.

The immune response and toxicity can be reduced by using "amplicons". HSV amplicons are prepared with a plasmid vector containing only the HSV origin of replication and appropriate packaging elements. The HSV sequence from the plasmid replicates and is packaged into virions by providing the viral genes *in trans*. [Spaete and Frenkel 1985]. The first generation of amplicons was obtained using a temperature-sensitive mutant of HSV-1 as helper virus [Geller and Breakefield 1988]. The resulting stocks were contaminated with helper virus, which expressed some viral genes and furthermore was able to revert to wild-type at high frequency. It caused the death of 10% of the treated animals which harbored signs of encephalitis [During *et al.* 1994] and striatal necrosis [Pakzaban *et al.* 1994].

"True helper-virus free" amplicons have been produced by using multiple restriction fragments of the helper virus genome lacking the packaging signals [Fraefel *et al.* 1996].

Amplicons have a large cloning capacity allowing insertion of multiple genes and transcription units. Furthermore, when smaller than wild-type HSV, amplicons encapsidate several copies of the genome, providing a higher level of expression of the transgene.

As HSV recombinant vectors, HSV amplicons can direct gene expression in neurones and glia. However, using HSV-amplicons, the duration of transgene expression was still less than 1 month [Linnik *et al.* 1995]. Cellular promoters appear to yield longer-term expression than the viral promoters [Kaplitt *et al.* 1994b]. Transactivation systems were tentatively used in order to increase transcription efficiency even further (for example, [Oligino *et al.* 1996]).

An interesting approach to enhance the duration of transgene expression was proposed by Costantini and collegues. [Costantini *et al.* 1999]. They constructed a hybrid HSV-AAV vector, which consists in an HSV amplicon containing an AAV vector, i.e. AAV ITRs flanking the transgene expression cassette, and AAV rep proteins. This hybrid vector achieved transduction in the rat striatum up to 1 month post-infection and in primary cultures from rat fetal ventral mesencephalon over a period of 12 days *in vitro*.

Retrograde axonal transport, i.e. migration of the viral particles intracellularly to the cell cytoplasm from the nerve terminals, is an interesting property of HSV vectors. It can be used to target diseased brain region by injection of vectors at distant sites. However, it also raises the risk of transport to undesirable regions and hence the question of safety in the clinics.

5.2.2. Adenoviral Vectors

Adenovirus (Ad) is a medium-sized non-enveloped virus having a 36-kb double-stranded DNA genome. This virus is associated with upper respiratory infections, eye infections and gastroenteritis in humans. Since the first demonstrations that Ad vectors can deliver and express transgenes when

injected directly in the brain, [Akli *et al.*, 1993; Bajocchi *et al.*, 1993; Le Gal La Salle *et al.*, 1993] these vectors have been evaluated in different models of NDD. They provide valuable information regarding fundamental aspects of gene delivery to the brain as well as potential therapeutical strategies.

Ad vector stocks with a very high titer (10^{11} to 10^{12} plaque-forming units/ml) can be produced with current protocols. Ad genome does not integrate in the host cellular genome. However, in non-dividing cells long-term gene expression can be achieved as the vector sequences remain episomal in transduced cells. As HSV, Ad is retrogradely transported. This useful property allows the protection of both the nerve terminals by infection of, e.g. surrounding muscle cells resulting in local secretion of the NF and the neuron cell bodies in the related structure after retrograde transport. Clinically, especially in the case of motorneurone diseases, this means protecting or even curing the neurones without entering the CNS.

The neuroprotective effects of an Ad vector encoding GDNF (Ad-GDNF) against 6-OHDA toxicity have been evaluated in two different paradigms. The first demonstration of a protective effect [Choi-Lundberg *et al.* 1997] consisted in injecting the vector in the SNpc before injection of 6-OHDA in the striatum (progressive model)(see Fig. **2**, panel B). The vector was expressing GDNF resulting in reduction in the loss of dopaminergic neurones. However, this report did not demonstrate any effect on motor behavior. Another study [Bilang-Bleuel *et al.* 1997] exploited the retrograde transport of Ad [Akli *et al.* 1993]. Indeed, although the adenoviral vector was injected into the striatum, gene expression occurred both *in situ* in the striatum and, after retrograde transport of the vector, in the SNpc. As already pointed out, this strategy allows protecting both dopaminergic terminals and cell bodies. Consistently, the authors demonstrated both an increase in dopaminergic cell survival and an improvement in the motor function. The protection of terminals is thought to be important because rescued adult dopaminergic neurones are unable to re-establish connections with the striatum. The group of M. Bohn further extended this observation. In these studies, even nanogram amounts of GDNF delivered by the adenoviral vector were sufficient to promote neuroprotection [Choi-Lundberg *et al.* 1997].

At long term, reduction of the number of transduced cells has been observed with first generation adenoviral vectors probably due to toxicity and/or vector-mediated immune response. Furthermore, demyelination caused by persistent adenovirus-induced immune responses has been documented [Byrnes *et al.* 1996a; Dewey *et al.* 1999]. The association of T cell-mediated inflammation with autoimmune diseases of the brain furthermore raises the possibility of anti-CNS autoimmune responses.

In addition to indirect signs of toxicity reflected by the drop in the number of transduced cells, reduction of the size of the striatum was reported following injection of both Ad-GDNF and Ad-lacZ viruses [Bajocchi *et al.* 1993; Bemelmans *et al.* 1999]. It is possible that capsid proteins or products from adenoviral genes present in the vectors are toxic to neurones. Neuronal and astrocytic cell death accompanied by positive TUNNEL assay after *in vivo* injections of Ad vectors has been documented [Thomas *et al.* 2001a]. A further argument in favor of this hypothesis is the observed toxicity of these vectors in neuron cell culture [Caillaud *et al.* 1993; Wilkemeyer *et al.* 1996; Easton *et al.* 1998].

Ad vector-elicited immune responses are probably at least partially responsible for the observed reduction of the number of transduced cells over time. In peripheral tissues, a biphasic immune response to adenoviral vectors has been described: 1) an early capsid-dependent inflammatory response characterized by multiple chemokine expression and acute tissue injury; 2) a cellular and humoral immune response against virus and transgene products [Yang *et al.* 1995a; Yang and Wilson 1995; Yang *et al.* 1995b;Yang *et al.* 1996; Yang and Wilson 1996]. Although the brain is an immune-privileged site, immune and inflammatory responses to adenoviral vectors also occur. Gene expression in the brain persists for longer periods than in the periphery but it is nevertheless limited by vector-mediated immune response and toxicity. The duration of gene expression is dependent on the target area, the amount of vector injected [Hermens and Verhaagen 1997; Bohn *et al.* 1999; Thomas *et al.* 2001a], and the degree of contamination with wild-type virus. During the early inflammatory phase, starting 12 to 24 h after injection, macrophages were demonstrated around the injection site as were as T-cells and monocytes along the blood vessels and upregulation of MHCI on microglia and vascular endothelium [Byrnes *et al.* 1995]. Concomitantly vector-driven gene expression decreased [Hermens and Verhaagen 1997]. Treatment with dexamethasone reduced inflammation and led to more persistent transgene expression in astrocytes but not in neurones [Hermens and Verhaagen 1998]. The early phase of inflammation is not affected by T-cell depletion and does not require vector-driven gene expression as demonstrated by the use of UV-irradiated virus [Byrnes *et al.* 1996b]. During the second phase, infiltration of T-cells and macrophages and further activation of microglia around the injection site were observed. This response subsides within 1 to 2 months while vector-driven gene expression can be detected for at least 2 months, suggesting that the virus is not cleared by the immune response [Byrnes *et al.* 1995]. This latter phase was not seen in response to UV-irradiated virus, and was therefore dependent on gene expression. The reduction of the number of transduced neurones in function of the time after injection of Ad vectors seems thus not to be related to a T-cell-mediated immune response. Recently, Thomas and collaborators [Thomas *et al.* 2001a] have shown that the decline in transgene expression is due to both direct vector-mediated acute toxicity and chronic inflammatory response.

A way to augment therapeutic effects without augmenting adverse effects is to use strong promoters allowing to obtain a higher level of gene expression while keeping the number of viral particles below the toxicity threshold [Gerdes *et al.* 2000]. These observations are crucial for the development of clinical protocols.

Even high doses of vector injected in the striatum do not induce neutralising antibodies [Thomas *et al.* 2001a].

Contrarily, peripheral exposure to the vector triggers renewed inflammation, infiltration of T-lymphocytes, activation of microglia [Byrnes *et al.* 1996a] and drastic elimination of transduced cells in the brain. This immune response is triggered by vector-driven gene expression and not by the capsid, as shown by the absence of immune response induced by peripheral challenge with a psoralen-treated adenoviral vector [Thomas *et al.* 2001b].

Recently developed "high-capacity" (HC) adenoviral vectors devoid of all viral genes [Schiedner *et al.* 1998] show promises toward non-toxic and stable gene delivery into the CNS [Thomas *et al.* 2001b]. Indeed, second phase chronic inflammation accompanied by massive infiltration of T-lymphocytes, microglial activation and up-regulation of MHC I expression does not occur when using HC-Ad. Furthermore, peripheral challenge does not reduce transgene expression in the brain. However, acute toxicity and inflammation, not related to gene expression, are identical to those obtained with replication-defective adenoviral vectors and the decline of transgene expression in the absence of peripheral challenge is comparable.

5.2.3. AAV

Adeno-associated virus (AAV) is a small human parvovirus having a 4.7-kb linear single-stranded DNA. Eight seroptypes have been cloned of which serotype 2 of AAV (AAV2) was cloned first and is most commonly used to derive vectors.

Various regions of the brain have successfully been transduced by recombinant AAV2 (rAAV2) vectors [Kaplitt *et al.* 1994c; McCown *et al.* 1996; Peel *et al.* 1997; Tenenbaum *et al.* 2000] with apparently no toxicity. In the striatum, rAAV2-mediated gene expression under the control of the CMV promoter was shown to be maintained up to 1 year [Mandel *et al.* 1998;Tenenbaum *et al.* 2000]. Little is known about the mechanism underlying the stability of rAAV2-mediated gene expression in the brain. Vector sequence integration is one possibility [Wu *et al.* 1998]. However, episomal sequences maintained at long term and relative absence of immune rejection, as observed in the muscle [Xiao *et al.* 1996] might also provide durable transduction [Lo *et al.* 1999]. After one month, however, a gradual decline in the number of transduced cells is generally observed [Klein *et al.* 1998]. The reduction in the number of transduced cells observed at long term when using the CMV promoter seems to be related to promoter extinction rather than to the loss of transduced cells or of viral sequences. Indeed, the use of neuron-specific enolase (NSE) promoter instead of the CMV promoter resulted in no decrease in transgene expression up to 3 months after injection of the vector, the latest time point shown [Klein *et al.* 1998].

Transduction efficiency in the striatum is low and limited to the vicinity of the delivery site. Multiple injections [Kirik *et al.* 2000b] and convection enhanced delivery [Bankiewicz *et al.* 2000] have been proposed to increase the size of the transduced area. More recently, co-infusion of mannitol [Mastakov *et al.* 2001] or heparin [Mastakov *et al.* 2002a] together with rAAV2 was shown to result in more widespread transduction in the striatum. The latter data suggests that partial occupancy of heparan sulfate proteoglycans (which serve as a receptor for AAV2; see further) by heparin results in less trapping of viral particles in the immediate vicinity of the delivery site, thus allowing spreading to more distant areas.

In addition to the differences in transduction efficiency, the kinetics of gene expression seem to vary depending on the site of injection [McCown *et al.* 1996; Klein *et al.* 1998]. For example, in the SNpc and in the globus pallidus, transduction efficiency raises its optimum earlier than in the striatum[McCown *et al.* 1996;Tenenbaum *et al.* 2000]. Since AAV is a single-stranded DNA virus, the target cell must allow the conversion of the single-stranded viral genome into a transcriptionally active double-stranded intermediate [Ferrari *et al.* 1996; Fisher *et al.* 1996]. Since uptake and migration of the viral particles to the nucleus is rapid [Bartlett *et al.* 1998; Seisenberger *et al.* 2001)] the delay required for transgene expression probably reflects the limited capacity of the cell to complete second-strand synthesis. Indeed, gamma irradiation, which is known to stimulate second-strand synthesis, results in earlier transgene expression in endothelial cells of the brain [Alexander *et al.* 1996]. An interesting approach to overcome the limitation of cell-mediated conversion to double-stranded rAAV DNA is the use of sel-complementary vectors [McCarty *et al.* 2001] which exploit the occurrence of dimeric tail-to-tail replicative intermediates. Indeed, using vectors having half (or less) of the wild-type AAV size, the dimeric forms of the recombinant genome being within the limits of packaging capacity, are encapsidated. Upon infection of a target cell, the dimeric tail-to-tail single-stranded genome can fold on itself and generate a double-stranded vector without the requirement for cellular factor-mediated DNA synthesis. The restricted insert size capacity (2.3 kb) of self-complementary vectors will nevertheless limit their usefulness.

Vectors based on rAAV2 having genes under the control of the CMV promoter preferentially transduce neurones [Klein *et al.* 1998; Bartlett *et al.* 1998]. Several explanations have been proposed for this cellular tropism. One is that CMV promoter is preferentially active in neurones [Baskar *et al.* 1996]. However, in contrast to rAAV2 vectors, Ad vectors with CMV promoter transduce all major cell types present in the brain [Akli *et al.* 1993; Le Gal *et al.* 1993; Bajocchi *et al.* 1993; Davidson *et al.* 1993]. The high multiplicity of infection used with Ad vectors could result in detectable expression of transgenes in cells in which the CMV promoter is less active than in neurones. However, very low multiplicities of infection of recombinant adenovirus harboring the mouse CMV promoter result in preferential transduction of glial cells [Gerdes *et al.* 2000]. We have compared the cell-type specificity of rAAV2 and rAd containing the CMV-EGFP cassette in the globus pallidus and shown that rAAV exclusively transduced cells with a neuron-like morphology while rAd mainly transduced cells with a glial-like morphology [Tenenbaum *et al.* 2000].

A more plausible explanation for the neuronal cell tropism of rAAV2 vectors is a more rapid uptake of the virus by neurones [Bartlett *et al.* 1998]. Indeed, in regions devoid of neurones, glial cells could be transduced using either the myelin-basic protein promoter specifically active in differentiated oligodendrocytes [Chen *et al.* 1999] or the

CMV promoter [Tenenbaum *et al.* 2000]. This suggests that the virus is able to transduce glial cells but in a complex cellular environment it is preferentially taken up by neurones.

The inability of the vector to enter specific cells due to the lack of its receptors, which mediate virus binding and entry, is one of the limiting steps for efficient gene delivery. AAV2 uses a cell surface heparan sulfate proteoglycan (HSPG) as a primary attachment receptor [Summerford and Samulski 1998] and fibroblast growth factor receptor (FGFR) as a coreceptor for entry into the host cells [Qing *et al.* 1999]. It has also been proposed that $\alpha V\beta 5$ integrin may have a role in AAV2 infection and could thus also act as a coreceptor for rAAV2 [Summerford *et al.* 1999].

The distribution of AAV2 receptor and co-receptors in the brain is poorly characterised. Two monoclonal antibodies specific for HSPG-related epitopes have been raised [David *et al.* 1992] and used in order to determine the cellular distribution of HSPG in the rat brain [Fuxe *et al.* 1994]. This study revealed a high immunoreactivity in some brain regions where efficient rAAV2-mediated gene transfer has been reported i.e. the hippocampus and the SNpc. Furthermore, the study by Fuxe and colleagues. [Fuxe *et al.* 1994] did not show immunoreactivity in the striatum where the efficiency of transgene expression has been reported to be low [McCown *et al.* 1996; Tenenbaum *et al.* 2000]. However, it seems likely that HSPG have a more widespread distribution in the brain than described by Fuxe and collegues. Indeed, there are some regional differences in the structure of the HSPG and in addition, posttranscriptional processing in terms of sulfatation and deacetylation reactions may have some effect on the recognition of the epitope by the mAb [Fuxe *et al.* 1994].

The localisation of FGFR1 in the rat brain has been investigated by immunohistochemistry using polyclonal antibodies [Matsuo *et al.* 1994]. This study revealed obvious correspondence between the brain regions in which efficient rAAV2-mediated gene expression has been reported [McCown *et al.* 1996; Klein *et al.* 1998] and the distribution of FGFR1. First of all, the FGFR1 immunoreactivity was present in neurones [Matsuo *et al.* 1994]. Interestingly, SNpc exhibited the most intense staining in the brain where the immunoreactivity appears to be preferentially localized in dopamine synthesizing neurones [Matsuo *et al.* 1994].

If the virus can actually enter the cell, subsequent intracellular trafficking and nuclear uptake [Duan *et al.* 1999; Bartlett *et al.* 2000; Hansen *et al.* 2001; Seisenberger *et al.* 2001] can be impaired and thus rate limiting.

Retrograde transport of rAAV2 viral particles from the delivery site in the striatum to the SNpc with efficient gene expression in TH-positive neurones has recently been demonstrated [Kaspar *et al.* 2002]. Previous studies failed to detect gene transfer of projection neurons after delivery to axons terminals [Peel *et al.* 1997; Klein *et al.* 1998; Mandel *et al.* 1999a; Tenenbaum *et al.* 2000]. This conflicting data could be explained by the higher viral titer (5×10^{10} infectious particles par ml) used by Kaspar and collaborators. Furthermore, transduction at distance from the injection site in the striatum, notably in the globus pallidus (GP) has been observed [Tenenbaum *et al.* 2000]. This raises the question of the risk of transgene delivery in undesirable regions.

As described in the studies with Ad vectors, rAAV2-mediated GDNF delivery in SNpc results in cell protection but not in behavioral improvement [Mandel *et al.* 1999b]. In contrast, rAAV2-mediated GDNF delivery in the striatum results in both an increase in the number of dopaminergic neurones and an improvement of rotational behavior [Kirik *et al.* 2000b]. In this study, GDNF delivery in the SNpc was even detrimental for behavioral recovery since injection of vector in both striatum and SNpc was less efficient than injection in the striatum alone. This could be due to the aberrant sprouting of dopaminergic neurones in SNpc in response to GDNF, which hampers sprouting of retracting fibers towards the striatum (for a discussion, see [Björklund *et al.* 2000]).

As discussed for Ad, immune responses against rAAV2 vary in function of the route of administration [Xiao *et al.* 2000; Brockstedt *et al.* 1999]. In the brain, antibodies to AAV2 capsid and transgene product appeared at low levels at 2-4 months after injection but did neither correlate with a reduction in transgene expression nor prevented the readministration of vector [Lo *et al.* 1999]. Accordingly, Mastakov and collaborators [Mastakov *et al.* 2002b] found that after unilateral administration of rAAV2 vectors, the same vector infused in the contralateral hemisphere after 3 months resulted in a transduction efficiency similar to that of a single injection. In contrast, when the interval between the 2 injections was 2 or 4 weeks a reduced transgene expression was observed. Interestingly, readministration of a second vector of a different serotype (rAAV5) or surprisingly of the same serotype but with a different genome resulted in the same transduction efficiency.

The recent cloning of vectors derived from different AAV serotypes, using different receptors [Xiao *et al.* 1999; Chiorini *et al.* 1997;Chiorini *et al.* 1999; Rutledge *et al.* 1998; Gao *et al.* 2002] showed promises for new developments of rAAV-mediated gene transfer in the brain. Indeed, vectors derived from AAV types 4 and 5 show patterns of transduction in the CNS, which are distinct from AAV2 [Davidson *et al.* 2000]. Recombinant AAV4 appears to transduce almost exclusively ependymal cells after injection in the striatum [Davidson *et al.* 2000] whereas rAAV5 transduces both astrocytes as well as neurones in wider area of the striatum than rAAV2 [Mastakov *et al.* 2002a].

Currently developed methods for capsid modifications (by mutagenesis [Rabinowitz and Samulski 2000; Wu *et al.* 2000], peptide insertion [Girod *et al.* 1999]and immunological re-targeting [Bartlett *et al.* 1999], etc.) will open new routes for specific targeting of particular brain regions and cell types. The recent determination of AAV capsid structure [Xie et al., 2002] is likely to further stimulate this field of research.

The main limitation of rAAV vector technology resides in the low cloning capacity (4.5 kb). The understanding of

the structure of concatemeric intermediates occurring in the process of rAAV-mediated transduction [Yang *et al.* 1999a] led to the development of *trans*-splicing vectors, which expand the utility of AAV vectors to larger inserts. However, this strategy requires the use of two separate vectors and will work only if the m.o.i is high enough to ensure a sufficient level of coinfection with both vectors after *in vivo* delivery and therefore requires very high titer preparations of rAAV to be produced. The size limitation is likely to be drastic in particular for efficient regulation, an issue that is likely to be important for the emergence of vectors that meet safety requirements. [Rendahl *et al.* 1998; McGee Sanftner *et al.* 2001a; Fitzsimons *et al.* 2001; Chtarto et al., 2002]. Similarly, cell type-targeting of transgene expression requiring the use of cellular tissue-specific promoters [Klein *et al.* 1998; Chen *et al.* 1999; Peel and Klein 2000; Paterna *et al.* 2000); Xu *et al.* 2001], generally larger than viral promoters, is expected to be limited by the size restrictions.

5.2.4. Lentiviral Vectors

Oncoretroviruses cannot cross the nuclear membrane and therefore require mitosis for integration in the chromosome of the host cell. In contrast lentiviruses, a family of complex retroviruses such as HIV, can integrate and replicate in nondividing cells since the preintegration complex is actively transported through the nucleopore [Lewis *et al.* 1992; Bukrinsky *et al.* 1992; Gallay *et al.* 1996]. This interesting property has led to the development of lentiviral vectors for gene transfer with a cloning capacity of 9 kb [Naldini *et al.* 1996a]. Lentiviral vectors are devoid of the HIV envelope and of sequences encoding viral proteins.

Most of the efforts so far have been focused on the development of efficient vector systems based on the pathogenic HIV-1 virus. The concern for biosafety has guided recent improvements in lentiviral vector technology. In the first generation of vectors, the gene encoding the HIV envelope has been deleted and the viral particles are pseudotyped with the envelope of vesicular stomatitis virus (VSV). As a result, HIV particles cannot be generated by recombination in the producer cells while the VSV envelope increases the tropism of the vector and the physical stability during concentration. In the second generation of vectors, the genes encoding the accessory factors of HIV that are associated with pathogenicity (nef, vif, vpr and vpu) have been deleted from the packaging construct [Zufferey et al. 1997]. With the third generation, tat-independent production of vectors was obtained [Dull et al. 1998]. The development of self-inactivating lentiviral vectors (SIN) in which the U3 region of the 3' long terminal repeat has been deleted resulting in inactivation of the viral promoter substantially improved the biosafety of HIV-1-derived vectors [Zufferey et al. 1998; Miyoshi et al. 1998]. These modifications reduced the risk of appearance of replication-competent viruses through recombination. Furthermore the genome has been split into four plasmids to further limit the formation of replication competent particles [Dull et al. 1998].

Alternative vector systems based on equine or feline lentiviruses, which appear to be non-pathogenic for humans, may be attractive candidates for clinical use [Curran *et al.* 2000;Mitrophanous *et al.* 1999;Poeschla *et al.* 1998], although it is still under debate whether these humanised non-primate vectors are safer than the HIV-derived vectors.

Lentiviral vectors (LV) efficiently transfer genes in adult rat brain with stable expression for several months [Naldini *et al.* 1996b;Naldini *et al.* 1996a; Blomer *et al.* 1997; Baekelandt *et al.* 2002; Bensadoun *et al.* 2000; Kordower *et al.* 2000]. Transgene expression can further be boosted using regulatory elements such as the woodchuck hepatitis virus posttranscriptional regulatory element [Donello *et al.* 1998; Zufferey] or the central polypurine tract and central termination site (cPPT/CTS) sequence which increases nuclear import of HIV-1 genomes [Follenzi *et al.* 2000; Zennou *et al.* 2000]. LV preferentially transduce neurones but also the glial cells [Baekelandt *et al.* 2002]. No significant inflammatory or immune response has been observed in the first studies [Blomer *et al.* 1997]. However, antibodies to viral proteins were present in the serum of immunocompetent mice but these did not interfere with the successful readministration of LV in the contralateral hemisphere. Furthermore, the efficiency and duration of gene transfer was similar in immune-competent and in severe combined immunodeficiency (scid) mice [Baekelandt, et al. 2002].

Ongoing improvements in LV technology that may be interesting for applications in the central nervous system include pseudotyping with neurone-specific envelope proteins [Beyer *et al.* 2002; Mazarakis *et al.* 2001; Watson *et al.* 2002] and development of tetracycline-regulatable LV[Vigna *et al.*, 2002].

LV have already been used in several neuroprotection studies in animal models of CNS diseases, like PD [Bensadoun *et al.* 2000;Deglon *et al.* 2000;Rosenblad *et al.* 2000b; Hottinger *et al.* 2000; Kordower *et al.* 2000]. Like Ad- and AAV-based vectors, HIV vectors expressing GDNF (LV-GDNF) injected in the SNpc could protect dopaminergic neurones from 6-OHDA toxicity [Bensadoun *et al.* 2000] as well as medial forebrain bundle axotomy [Deglon *et al.* 2000]. In the latter study, the number of TH-positive cells in the SNpc was significantly enhanced in the GDNF-injected rats as compared to the lacZ group [Deglon *et al.* 2000]. However, the dopamine level in the striatum was identical in the 2 groups, probably reflecting the lack of GDNF-stimulated reinnervation when GDNF is administered in the SNpc. In contrast, after injection of LV-GDNF into the striatum [Georgievska *et al.* 2002] or into both the striatum and SNpc [Rosenblad *et al.* 2000b], the striatal TH-immunoreactive innervation was partly spared. Injection of LV-GDNF into the striatum and SNpc of nonlesioned aged rhesus monkeys augmented dopaminergic function. In young adult rhesus monkeys treated one week before with MPTP, LV-GDNF prevented nigrostriatal degeneration and reversed motor deficit in a hand-reach task in 3 out of 4 animals [Kordower *et al.* 2000]. In addition to the nigral neuron protection, the behavioral improvement could furthermore be related to the fact that the low number of TH-positive neurons naturally present in the primate striatum was 8-fold increased after LV-GDNF delivery [Palfi *et al.* 2002]. In this study, two monkeys died for unknown reason. Post-mortem histopathological analysis revealed mild hepatic necrosis. Furthermore, immunohistological analysis of brains from

LV-treated animals revealed mild staining for activated microglia and leucocytes in some but not all animals.

5.3. Non-Viral Vectors

Non-viral vectors are an attractive alternative to viral vectors because of their safety and lack of immunogenicity. However, their toxicity might limit their usefulness in the field of NDD.

In non-dividing cells, in particular that of the nervous system, transport of DNA to the nucleus is a major limiting step for non-viral DNA delivery [Dean 1997]. Therefore, gene transfer using liposomes has generally failed in the brain. Consistent with transport to the nucleus being a limiting step, the use of the bacteriophage T7 promoter in combination with RNA polymerase, a cytoplasmic gene expression system allowed to obtain a significant gene expression in the brain [Mizuguchi *et al.* 1997]. However, the short duration of gene expression in this study (about 24 hours) was not compatible with current strategies for NPGT.

DNA sequences enhancing transport to the nucleus can be introduced into the DNA constructs in order to facilitate liposome-mediated gene transfer into the brain. SV40 virus origin of replication [Dean 1997] as well as tissue-specific promoter contains sequences, which localise to the nucleus of the cells in which they are active [Mizuguchi *et al.* 1997]. These clues suggest strategies to facilitate the nuclear import of plasmid DNA.

In lesioned brain, however, transgene expression after injection of DNA complexed to liposomes could be obtained. In 6-OHDA treated animals, TH was expressed in astrocytes under the control of the GFAP astrocyte-specific promoter [Segovia *et al.* 1998]. In a subsequent study using 6-OHDA treated animals, TH and AADC were expressed and significant recovery of parkinsonian animals was observed up to 5 weeks after injection of the liposome:DNA complexes. In this study, the bovine-papilloma virus origin of replication and replication genes were incorporated in the plasmid in order to prolong the transgene expression. However, continuous injection of complexes during 7 days was necessary [Imaoka *et al.* 1998] to obtain these results.

6. COMBINING CELL REPLACEMENT AND GENE THERAPY

Cell replacement therapies such as transplantation of foetal mesencephalon could be advantageously combined with neuroprotective gene therapy in two ways. First, cell replacement does not slow down the progression of the disease. Therefore protection of remaining host cells is likely to be of great benefit even when grafted cells with similar function compensate the neuronal loss. Second, grafts themselves are submitted to oxidative stress and apoptosis and neuroprotective gene transfer could improve graft survival and functionality.

Grafts of foetal mesencephalon cell suspensions or solid fragments in patient's putamen partially ameliorate motor functions presumably through re-establishment of dopaminergic activity [Lindvall 1997]. Clinical improvements appear to be related to the survival and functionality of transplanted tissue as shown by post-mortem analysis of brain tissue [Remy *et al.* 1995; Hagell *et al.* 1999]. The poor survival of grafted cells, evaluated as 5-10%, together with the limited availability of the human foetal tissue are important limiting factors for both the success and the widespread applicability of transplantation therapy.

In animal models of PD, foetal nigral transplants have been shown to survive in the striatum, provide striatal reinnervation and improve motor function. However, most implanted embryonic neurones die within a week posttransplantation. Intrastriatal administration of GDNF prior to transplantation promotes the protection of grafted cells [Rosenblad *et al.* 1996; Sinclair *et al.* 1996]. Storage of DA cells in GDNF (1mg/ml) at 4°C for 6 days prior to transplantation promotes grafts survival in rats [Apostolides *et al.* 1998] and in patients [Mendez *et al.* 2000]. However, the short half-life together with the undesirable effects of high doses of neurotrophic factors complicates their use as recombinant proteins in transplantation therapies.

Lipid-mediated, transient expression of GDNF (up to 176 pg/ml) in the graft promotes survival and results in better recovery of grafted animals [Bauer *et al.* 2000]. Thus, using a gene transfer method, 10^7-fold less GDNF is sufficient to produce a functional effect. However, lipid-mediated transfection can only be performed in culture, which necessitates an additional manipulation to the tissue. It has been shown that the tissue can be maintained up to one week in culture without reduction in the number of DA neurones or modification of the graft efficiency [Hoglinger *et al.* 2001]. However, transfection, even if it allows sufficient expression of GDNF at short-term to enhance graft's survival, does not provide long-term GDNF delivery in the damaged brain to promote protection and rescue of host dopaminergic neurones.

Viral vectors, which mediate sustained gene expression would allow, in addition to counteract acute cell death during transplantation, to increase neurite extension from the graft and to deliver GDNF to the host. Gene transfer to dissociated foetal dopaminergic cells mediated by first generation Ad vectors has been described [Barkats *et al.* 1997]. However, Barkats and colleagues. demonstrated a toxic effect of Ad vectors in the grafts as well as a drop in transgene expression with time. Costantini and collaborators [Costantini *et al.* 1999] confirmed the toxicity of adenoviral vectors.

Gene delivery to foetal dopaminergic neurones using HSV-based vectors as well as HSV amplicon vectors and hybrid herpes/AAV amplicon vectors [Costantini *et al.* 1999] has also been recently attempted. In the same study, AAV vectors did not transduce dopaminergic cells. The levels of both adenoviral and HSV vector-driven transgene expression dropped at 12 days post-infection as compared to 2 days post-infection. In contrast, hybrid HSV/AAV amplicon vectors containing the AAV rep open reading frame mediated efficient gene expression that was maintained up to 12 days post-infection, in the absence of significant toxicity.

We have shown that an AAV vector can mediate the expression of the EGFP reporter gene under the control of the CMV promoter in organotypic cultures of freshly explanted solid fragments of human foetal mesencephalic tissue from 3 days to at least 6 weeks post-infection (see Fig. 5) [Tenenbaum *et al.*, 2000][2]. The number of transduced cells increased with time, as already observed in adult CNS. Using rat tissue, it was furthermore demonstrated that transduction remained stable until at least 3 months posttransplantation [Lehtonen *et al.* 2002]. The failure to observe AAV-mediated gene transfer in dissociated foetal DA cell cultures in the Costantini's study, possibly reflects the different conditions of infection.

This promising data suggests that AAV vectors expressing NF could be used to genetically modify the foetal tissue prior to transplantation in order to promote graft survival and integration. The graft, which was recently shown to be maintained for at least 10 years in patients [Piccini *et al.* 1999] is furthermore an excellent cellular vector to deliver NF to the host in order to provide long-term neuroprotection and possibly halt the progression of the disease.

Stem cells or neuronal progenitors could provide an ideal source of cells for combined cell replacement and gene therapies. Embryonic stems cells have been shown to differentiate into functional dopaminergic neurones after transplantation into the striatum of 6-OHDA-lesioned rats [Bjorklund *et al.* 2002]. However, in 5 out of 19 animals, teratoma-like structures developed at the implantation site, suggesting that at least part of the transplanted ES cells continued to divide. Differentiating the embryonic stem cells *in vitro* prior to transplantation constitutes an interesting alternative. Indeed, enrichment of ES cell cultures in dopaminergic neurones using a five-stage differentiation method together with transfection with the Nurr1 gene (a transcription factor which is required for dopaminergic differentiation) resulted in grafts that were functional and did not give rise to tumors until at least 8 weeks after transplantation [Kim *et al.* 2002].Neural progenitors can be harvested from the subventricular zone of adult mammals [Gritti *et al.* 1999] or from various regions of fetal brain [Svendsen *et al.* 1998; Svendsen *et al.* 1997a] and expanded *in vitro*. The feasibility of transducing neural progenitors of fetal [Hughes *et al.* 2002; Wu *et al.* 2002] or adult [Falk *et al.* 2002] origin with viral vectors has recently been demonstrated. These pioneer studies have opened the door for the genetic modification of neural progenitors with the aim to drive their differentiation towards dopaminergic neurones as well as to enhance their survival after transplantation.

7. REGULATION OF GENE EXPRESSION

As described above, transgene expression in the brain has previously been demonstrated with a variety of different viral vectors in which constitutive promoters drive transgene expression. However, for clinical applications it is often desirable to limit this expression to a defined time frame and/or to precisely control its level. For example, excess of neuroprotective factors is likely to cause severe side-effects. The level and durability of gene expression need thus to be adjusted in order to minimize undesirable effects. Motor disturbances resulting from inappropriate expression of genes coding for NF in animal models have been described [Kirik *et al.* 2000b]. In this study AAV-mediated expression of GDNF in the right striatum of healthy animals resulted in an asymetrical behavior, presumably resulting from excess of dopamine in the injected side relative to the intact side. Lesioning of the right SNpc of the AAV-GDNF-treated animals by unilateral injection of 6-OHDA, restored a symetrical behavior (see Fig. 4). This experiment demonstrates that the amount and period of administration of NF should be adjusted by taking into account the extent of neurodegeneration.

Several promoter systems are available which are capable of regulating gene expression in eukaryotic cells ([Smith-Arica and Bartlett 2001] and references therein). These include promoters whose activity is altered in response to heavy metal ions [Mayo *et al.* 1982; Palmiter *et al.* 1983], isopropyl-β-D thiogalactoside [Baim *et al.* 1991] and hormones [Lee *et al.* 1981].

These endogenous systems usually suffer from the following problems : 1) the inducer evokes pleiotropic effects that complicate the analysis of the resulting phenotype, 2) toxicity of the inducing agents, and 3) a narrow window of induction [Gossen *et al.* 1993;Yarranton 1992]. Two systems have been developed that appear to overcome many of the problems associated with inducible vectors : the tetracycline (Tet) [Gossen and Bujard 1992] and progesterone antagonist (RU486) [Wang *et al.* 1994]. Both systems use microbial proteins and microbial DNA response elements to drive the expression of a desired gene in heterologous cells.

The Tet-Off system developed by Gossen and Bujard [Gossen and Bujard 1992] is composed of a Tet transactivator (termed the tTA), which, in the presence of tetracycline, binds the Tet-regulatable element (TRE) and initiates transcription from a minimal promoter. By modifying tTA by four amino acids, the Tet-On transactivator (termed rtTA) was generated [Gossen *et al.* 1995]. The rtTA was shown not to bind to the TRE in the absence of drug; however, in the presence of the Tet analog doxycycline (Dox), Tet-On binds to the TRE and activates transcription.

In clinical applications, the pharmacological properties of the inducer drug are a major concern. The antibiotic doxycycline is widely accepted, because of its safe use in humans, its pharmacological behavior, its specificity for the bacterial tetracycline repressor (TetR), and the dose of drug necessary to produce detectable protein product which is within the range acceptable for the clinical use [Solera *et al.* 1996]. Also, because of the small size of the transactivator expression cassette (2,1 Kb), the tetracycline inducible system could be employed in different viral vectors, in single autoregulatory cassette, including those based on adenovirus

[2] L. Tenenbaum, F. Bonnaud, M. Peschanski, C. Melas, G. Saber, A. Stathopoulos, F. Rodesh, T. Velu and M. Levivier. (1999) AAV-mediated transduction of human fetal mesencephalon. Society for Neuroscience Abstract n° 2126.

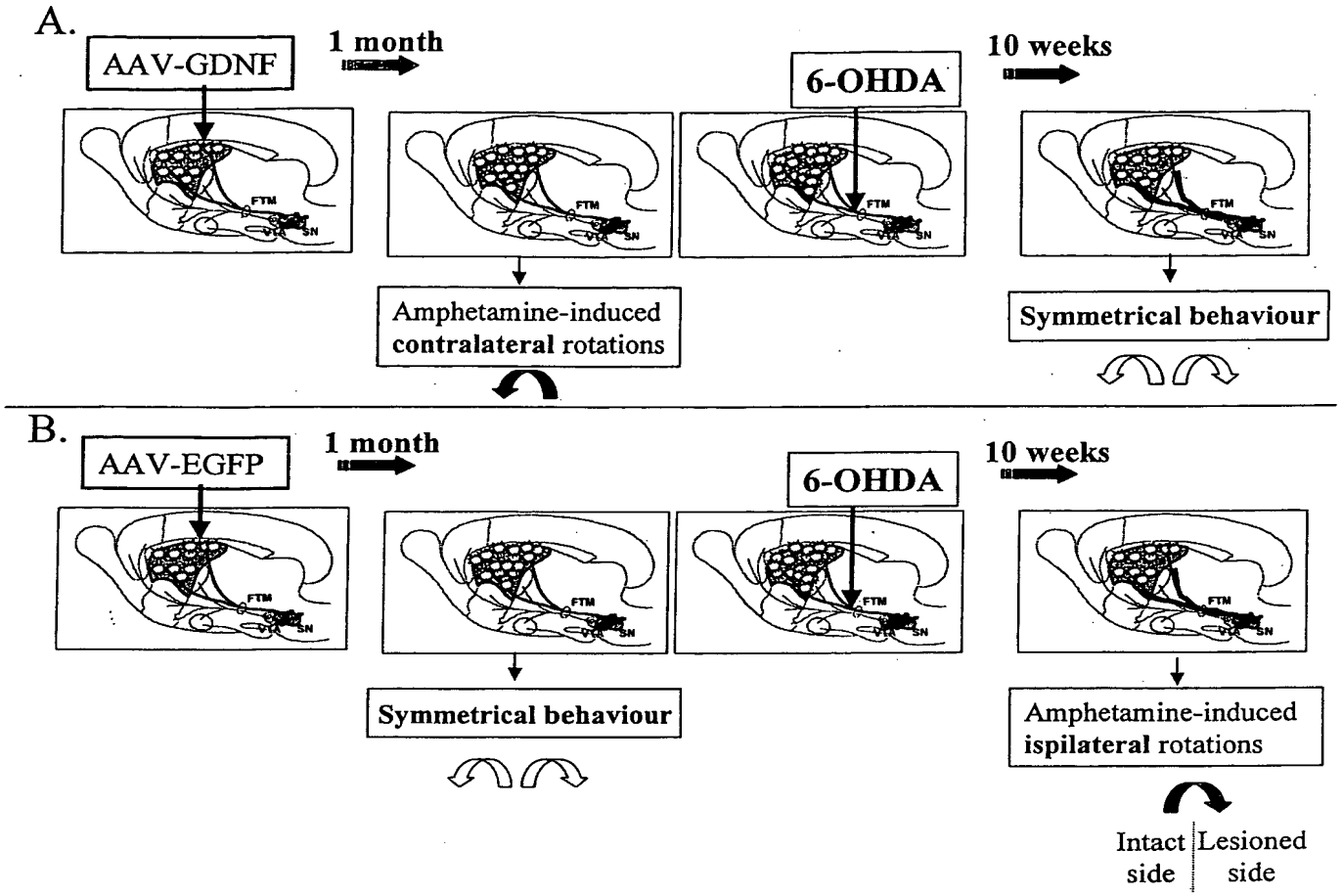

Fig. (4). Side effects of unappropriate expression of GDNF.

The neuroprotective effect of AAV-GDNF is evaluated by intrastriatal injection. The vector is injected 1 month prior to 6-OHDA administration in order to allow full expression of the transgene. Rotational behaviour is evaluated immediately before or 10 weeks after the injection of the toxin .

A. Four weeks after delivery of AAV-GDNF, contralateral rotations appear. Ten weeks after injection of the toxin, symmetrical behaviour is observed.

B. In the control group AAV-EGFP is injected instead of AAV-GDNF. Animals harbour an ipsilateral rotational behaviour in response to 6-OHDA.

Adapted from Kirik *et al.*, 2000a.

[Corti *et al.* 1999, adeno-associated virus [Haberman *et al.* 1998; Fitzsimons et al., 2001; Chtarto et al., 2002], retrovirus [Hofmann *et al.* 1996], and herpex simplex virus [Ho *et al.* 1996]. However, some of the existing regulatory systems based on doxycycline and the tetracycline repressor (TetR) have high basal background expression in the non-induced state and/or suffer from a low level of induction [Haberman *et al.* 1998;Saez *et al.* 1997; Ackland-Berglund and Leib 1995], which results in modest induction factors. Some strategies have been developed to overcome these problems.

Indeed, in a retroviral delivery system, there is an interference between the Tet regulation of the minimal promoter and the strong moloney viral 5' long terminal repeat (LTR) enhancer and promoter activity. The use of a SIN vector (self-inactivating vector) allows to avoid this interference. Following infection of the target cell, the SIN vector, which contains a deletion in the enhancer and promoter sequences of the 3' LTR, transfer this deletion to the 5' LTR, resulting in transcriptional inactivation of the provirus vector [Hofmann *et al.* 1996].

Similarly, an influence of the adeno-associated ITR upon the minimal promoter cassette of a tetracycline inducible system may increase basal activity [Haberman *et al.* 1998]. Indeed some evidence exists that the AAV ITRs have transcriptional activity, as either a promoter or an enhancer that may interfere with the regulated expression cassette. Since the AAV ITRs contain all cis-acting sequences necessary for replication and packaging of recombinant

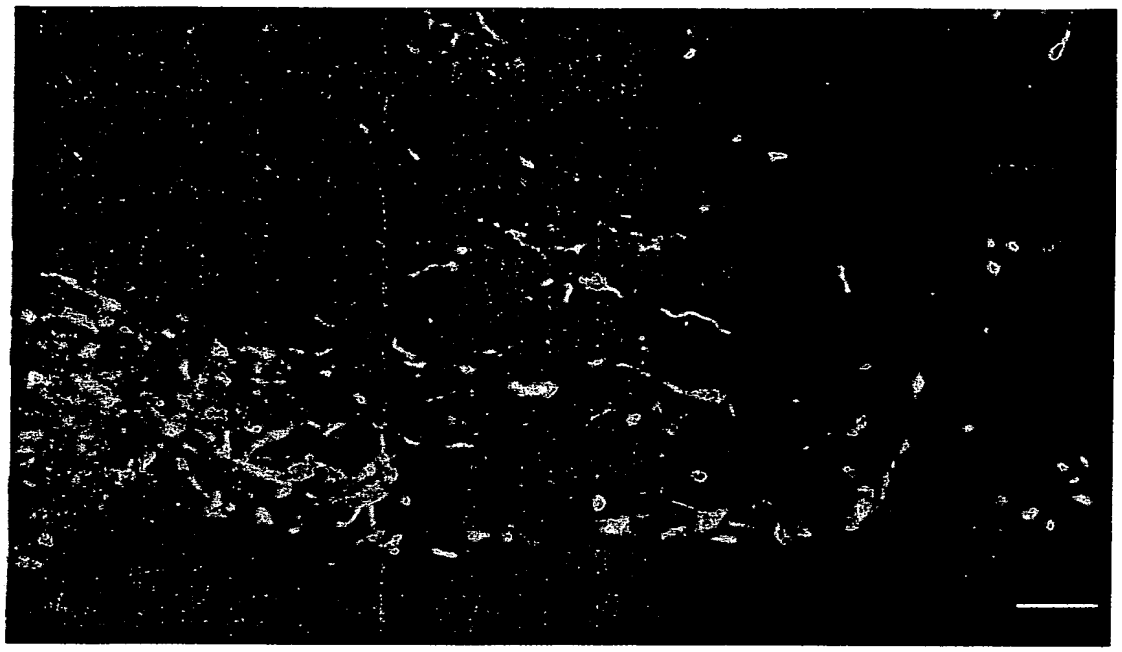

Fig. (5). rAAV-mediated gene transfer into human embryonic VM.

Pieces of embryonic VM were subjected to two 1-µl injections of rAAV-EGFP with a titer of 1.2 x 10^8 I.U./ ml. Pieces were maintained in organotypic culture until fixed and frozen 6 weeks postinfection. Twenty-micron "free-floating" cryostat sections were processed for GFP-immunolabeling. Bar corresponds to 100 µm.

DNA as well as for mediating the integration of the viral DNA into the host genome, they cannot be deleted [Haberman *et al.* 2000]. To prevent unregulated gene expression in the Tet-On system, an other component has been used. It encodes a transcriptional silencer (termed tTS) that bloks transcription of genes under control of the TRE in the absence of doxycycline [Witzgall *et al.* 1994]. Two constructs were made and packaged into rAAV particles: a silencer/activator vector and an inducible doxycycline-responsive vector [McGee Sanftner *et al.* 2001b]. We have recently described a single AAV vector in which transcription of both the reverse tetracycline transactivator (rtTA) and the transgene are initiated from a bidirectional tetracycline-responsive promoter and terminated at bidirectional SV40 polyadenylation sites flanking both ITRs [Chtarto et al., 2002] As compared to previously described autoregulatory tet-repressible (tetOFF) AAV vectors [Fitzsimons *et al.* 2001], the use of tet-inducible vectors avoid chronic antibiotic administration in the uninduced state.

The position and the orientation of the tetracycline-controlled elements in adenovirus as well as in some other viral vectors are also important for stringent gene regulation. It has been reported that the minimal CMV promoter was leaky when inserted into a recombinant adenovirus in a tail-to-tail orientation with the tTA transcription unit. Therefore, the transcription units encoding tTA and transgene were inserted head-to-tail and separated by the murine genomic sequence UMS (upstream mouse sequence) to limit transcriptional interference [Corti *et al.* 1999; McGeady *et al.* 1986].

The recombinant adenoviral vector has also been used to make a new generation of vectors, in which combined cell-type specificity and simultaneous regulation of transgene expression can be obtained in the brain. This was accomplished through the use of cell-type-specific promoters in combination with the tetracycline inducible system in dual adenoviral vector [Smith-Arica and Bartlett 2001].

8. CONCLUSIONS AND DISCUSSION ON STRATEGIES

Current pharmacological treatments for Parkinson's disease aim at raising the level of dopamine in the striatum, e.g. by oral administration of dopamine precursors or agonists. When the disease progresses, these treatments cease to be effective and provoke undesirable effects. At the later stages of the disease, neurosurgical interventions such as transplantation of foetal dopaminergic neurones in the striatum, or interference with the motor loop by electrical stimulation are proposed. All these treatments, whether pharmacological or neurosurgical are compensatory but do not affect the progression of PD. New therapeutic strategies based on neuroprotection could generate treatments, which can halt or reduce degeneration of dopaminergic neurones. The existence of a blood-brain barrier often precludes systemic delivery of neuroprotective factors. *In situ* gene delivery provides strategies for local and sustained

administration of these factors at physiologically relevant doses. However, the cellular and viral vectors available to achieve such gene transfer still require further improvements and characterisation.

Various cell types (immortalised cell lines, fibroblasts, endothelial cells, astrocytes, etc.) have been used as cellular vectors for *ex vivo* gene delivery in the brain. Although many of these led to significant behavioral recovery in the animal models, it is likely that ultimately stem cells, possibly of various tissue origins, will be the source for cell replacement and *ex vivo* gene delivery in human clinical trials (reviews: [Svendsen 2000;Svendsen and Caldwell 2000]). Identification of factors governing the survival and differentiation of grafted cells in the diseased brain (see for example: [Snyder *et al.* 1997; Svendsen *et al.* 1997b]) will be of overwhelming importance for the success of cellular therapies. Cell biology studies addressing these issues are still in their infancy. Furthermore, combination of cell replacement therapy with neuroprotective gene therapy through the use of neural stem cells expressing neuroprotective factors under appropriate transcriptional regulation constitutes a promising approach for the future. Fundamental virology has generated important discoveries, which have led and are likely to further lead to the development of new or improved viral vectors and methods for their production and purification. For example, immune and inflammatory responses as well as apoptotic cell death induced by replication-defective adenoviral vectors has raised the question of their usefulness for the treatment of NDD. Recently developed gutless adenoviral vectors deleted of all viral genes [Mitani *et al.* 1995; Kochanek *et al.* 1996; Hardy *et al.* 1997] elicit no chronic inflammation [Thomas *et al.* 2001b]. Nevertheless, immune responses can be elicited in the brain after peripheral challenge. Use of low doses of "gutless" vectors, in combination with strong promoters might help circumventing this problem [Gerdes *et al.* 2000]. However, the therapeutic dose of vectors to be applied to humans might be such that immune and inflammatory responses in the brain after opportunistic peripheral adenoviral infection cannot be avoid. Recently developed vectors based on canine adenovirus [Kremer *et al.* 2000;Soudais *et al.* 2001] are likely to be less immunogenic for humans and thus constitute an interesting alternative that deserves further characterisation.

Similarly, HSV amplicon vectors devoid of all HSV coding sequences [Saeki *et al.* 2001] and prepared using split helper plasmids to limit contamination with replication-competent HSV genomes, have allowed to obtain relatively long-term transgene expression in the brain [Costantini *et al.* 1999]. Among viral vectors used in the CNS, HSV amplicons have the largest cloning capacity. This property justifies further research to improve the system.

At present, AAV vectors are certainly the most promising system in terms of safety profile and durable gene expression. Their main limitation resides in the low cloning capacity. This limitation is likely to be drastic in particular for efficient regulation of transgene expression [Rendahl *et al.* 1998; McGee Sanftner *et al.* 2001a; Fitzsimons *et al.* 2001; Chtarto et al., 2002]. The understanding of the structure of concatameric intermediates occurring in the process of rAAV-mediated transduction [Yang *et al.* 1999b] has led to the development of trans-splicing vectors, which expand the utility of AAV vectors to larger inserts. However, this strategy again requires the use of two separate vectors. Transduction efficiency of the current vectors seems lower than that achieved using lentiviral vectors. However, the use of stronger promoters such as the CMV/chicken-β-actin hybrid promoter [Peel and Klein 2000] might result in higher efficiencies. Furthermore, vectors derived from other AAV serotypes might provide more efficient transduction of cell types that are poorly transduced by rAAV2 vectors [Davidson *et al.* 2000; Chao *et al.* 2000].

The extensive use of AAV vectors has been hampered by the difficulties in producing these vectors in large quantities. The discovery of the HSPG as a receptor for AAV [Summerford and Samulski 1998] led to the use of heparin columns [Zolotukhin *et al.* 1999] instead of CsCl gradients, allowing a higher recovery as well as a more scaleable production process usable in GMP production. The stability of AAV viral particles allowing a final filtration step [Drittanti *et al.* 2001] reduces the need for a highly sterile environment thereby reducing the costs of GMP production as compared to retroviral and lentiviral vectors. The knowledge of the adenoviral genes required for the AAV helper effect has led to the development of complementing plasmids, which can be used instead of adenovirus [Gao *et al.* 1998;Grimm *et al.* 1998] avoiding heat inactivation of residual adenovirus and resulting in high-titer stocks devoid of adenovirus. The pDG helper plasmid harboring a large heterologous promoter from the "mouse mammary tumor virus" replacing the p5 promoter [Grimm *et al.* 1998] is furthermore incapable of giving rise to wild-type AAV virions.

Lentiviral vectors efficiently transfer genes in adult rat brain with stable expression for several months [Blomer *et al.* 1997]. However, issues related to the safety of these vectors have not been fully addressed yet. In particular, potential inflammatory or immune response [Blomer *et al.* 1997] has not been studied in detail. Due to the pathogenicity of the HIV virus, most of the efforts so far have been focused on totally avoiding the formation of wild-type virus. In the first generation of vectors, the viral particles are pseudotyped with the envelope of vesicular stomatitis virus (VSV). As a result, HIV particles cannot be generated by recombination in the producer cells. In the later generations of vectors, the genes encoding factors that are associated with pathogenicity (nef, vif, vpr and vpu) have been deleted from the packaging construct [Zufferey *et al.* 1997]. The development of self-inactivating LV (SIN) substantially improved the biosafety of HIV-1-derived vectors [Zufferey *et al.* 1998; Miyoshi *et al.* 1998]. Furthermore the genome has been split into four plasmids to further limit the formation of replication competent particles [Dull *et al.* 1998]. However, it would be prudent to further evaluate LV regarding the biosafety aspects in animal models. LV should not be pushed too quickly into the clinic. If unexpected toxicity problems arise in treated patients, the future of this powerful new gene transfer technology would possibly be jeopardized.

Despite the above-described accumulating evidence that gene therapy of PD is not far from being ready for the clinics, several studies have demonstrated that the shift to the patient should be made with extreme caution to avoid straightforward failures, which would discourage further advances. An insufficient level of or a time-dependent reduction of gene expression could compromise a positive clinical output. The Further development of strong promoters of cellular origin which are not down-regulated in the CNS by methylation is still a matter of research. The immune and inflammatory responses to viral vectors in pathological situations, in particular when the blood-brain barrier might be disrupted, also deserve further studies. In this respect, new non-viral vectors could offer interesting alternatives. Finally, the main limitation of the widespread use of gene therapy is probably the high cost of the production and quality control of viral vector stocks. Therefore, research aimed at the discovery and development of non-viral vectors, which until now have not produced encouraging results in the field of gene transfer to the brain could, in the future, lead to the development of more marketable vectors for neuroprotective gene therapy.

The insults provoking dopaminergic cell death are poorly defined. In particular, it is not known whether the initial trigger takes place in the cell bodies or in the terminals. Therefore, optimally, neuroprotective factors should be present both in the SNpc and in the striatum. Retrograde transport of recombinant virus and/or of NF could allow to use the striatum, a site that is surgically more accessible than the SNpc, as a target for neuroprotective gene delivery. In some cases, however, the gene has to be present inside the cell that is to be protected (e.g. anti-apoptotic genes such as bcl-2).

The route of administration might also be a major limitation in the clinics. For example, the striatum is more accessible than SNpc. Developing imaging technologies for controlling the spatial localisation of gene expression in the brain, is an important challenge for the next few years. Clinical trials should not be initiated before fundamental research has allowed optimising the vectors and clearly identifying the risks inherent to their use in humans.

It is also of overwhelming importance to develop genetic elements allowing to control gene expression in terms of amount of gene product and period of expression in order to avoid undesirable effects which, in the context of the precise regulation of the motor loop, could be as invalidating as the disease symptoms.

In conclusion, is NPGT clinically feasible? Proofs of principle have been made. However, even when a clinical improvement is observed, one has to be very careful with the interpretation of the success. For example, animal experiments have repeatedly shown that surgery itself can induce a short-term neuroprotective effect (for example, sham effect [Przedborski *et al.* 1991]. Therefore, long-term clinical follow-up is very important for the evaluation of therapeutic effect.

Furthermore, severe side-effetcs could appear. Indeed, the motor loop is strictly balanced. Movement results from an intricate circuitry, the disturbance of which results in severe motor defects. Therefore, factors stimulating or inhibiting the functioning of neuronal cell populations involved in the control of movement have to be administered in a controlled way. Tight regulation of gene expression will thus have to be achieved before clinical trials can be initiated. Prokaryotic systems are likely to provide gene regulation induced by factors that have no effect on eukaryotic gene transcription (for example tetracycline). Another elegant possibility is to use cellular promoters, which are naturally induced in conditions of ongoing neurodegeneration. Metabolism-related gene expression has already been proposed in other organs [Inazawa *et al.* 2001].

Cooperation between virologists, neurobiologists and clinicians will be of overwhelming value in this field.

The evaluation of vectors safety as well as a better knowledge of nerve cell biology must guide clinical trials. Animal experiments with statistical number of animals need to be performed before clinical trials are initiated, especially in the case of PD for which other effective treatments exist.

ACKNOWLEDGEMENTS

We thank Dr Marc Peschanski for stimulating discussions.

E.L. and A.C. are recipients of fellowships from the Belgian National Research Foundation (F.N.R.S./ Télévie). D.B. is recipient of a post-doctoral fellowship from the Belgian National Research Foundation (F.N.R.S.). V.B. is a postdoctoral fellow of the Flemish Fund for Scientific Research (FWO Vlaanderen). This work was also supported by EC grant BIO-CT97-2207, by grants of the Belgian National Research Foundation (FNRS-FRSM) n° 3.4565.98 and n°3.4619.00 and by a grants from "Société Générale de Belgique" and "Bruxelles-Capitale".

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